4 Publikace

Kapilární elektroforéza v cyklujícím gradientu: nízkonákladová metoda pro vysokokapacitní analýzu genetických variací

Minarik M, Minarikova L, Bjorheim J, Ekstrom PO.

Electrophoresis. 2003 Jun;24(11):1716-22

V předkládané práci ukazujeme nový typ detekce DNA variací. Tato metoda představuje přechod od techniky využívající horizontální gelovou denaturační elektroforézu k multikapilární elektroforéze na DNA sekvenátoru za optimalizovaných podmínek umožňujících maximální počet analyzovaných vzorků a vysokou účinnost. Hlavní vylepšení je založeno na aplikaci cyklujícího teplotního gradientu namísto jednokrokového gradientu běžně používaného při denaturační elektroforéze. Toto vylepšení umožňuje použití techniky dávkování několika vzorků za sebou během jedné analýzy v definovaných časových intervalech. Periodická oscilace teploty poskytuje totožné separační podmínky pro všechny vzorky dávkované v jedné sérii. Použitím této nové techniky jsme docílili výrazného zvýšení počtu analyzovaných vzorků přeměnou standardního komerčního 96-ti kapilárního analyzátoru na mutační detekční systém schopný screeningu více než 15 000 vzorků za 24 hodin. To představuje desetinásobné zvýšení kapacity oproti současným srovnatelným technologiím.

Použití kapilární elektroforézy v cyklujícím gradientu pro detekci bodových mutací v genech APC, K-ras a DCC u pacientů se sporadickými kolorektálními tumory

Minarik M, Minarikova L, Hrabikova M, Minarikova P, Hrabal P, Zavoral M.

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V této práci byla použita dříve popsaná metoda kapilární elektroforéza v cyklujícím gradientu (CGCE) k monitorování molekulárních změn během přechodu adenomu ke karcinomu při progresi sporadického kolorektálního karcinomu. Cílem práce byla optimalizace separačních parametrů pro vybrané mutační oblasti tumor supresorových genů majících roli v časných stádiích kolorektální karcinogeneze a následné vyšetření těchto mutací v klinických vzorcích pacientů s adenomatózními polypy a časnými karcinomy. Celkem bylo vyšetřeno 47 kolorektálních tumorů v různých stádiích progrese. Hlavní důraz byl kladen na vyhodnocení senzitivity a specificity detekce mutací potřebné k odhalení časného stádia onemocnění. Celkem bylo nalezeno 7 různých typů somatických mutací u 32 vzorků s mutovaným genem K-ras a 1 vrozená a 5 somatických mutací v 15 vzorcích mutovaných v genu APC. V genu DCC nebyla nalezena žádná z dříve popisovaných mutací. Kromě jednoduché optimalizace separačních podmínek vykazuje metoda CGCE také vysokou senzitivitu a selektivitu umožňující detekci jednak malé mutační frakce a jednak kombinaci několika různých mutací v rámci jedné sekvence.

Multikapilární elektroforéza neznačených DNA fragmentů s vysoce citlivou detekcí laser-indukované fluorescence za použití protisměrné migrace interkalačního barviva

Benesova-Minarikova L, Fantova L, Minarik M.

Electrophoresis. 2005 Nov;26(21):4064-9

Analýza PCR produktů pro různé aplikace zahrnující screening nukleotidových polymorfismů, detekci somatických mutací nebo kvantifikaci produktů reverzní transkripce se stává základní metodou při klinickém výzkumu, preventivním testování, diagnostickém screeningu nebo farmakogenomickém genotypování. V současné době jsou při prevenci, diagnóze i léčbě genetických onemocnění (nádorových, kardiovaskulárních nebo neurodegenerativních) používány různé techniky založené na CE analýzách pomocí multikapilárních sekvenátorů. Jedním z hlavních faktorů hrajících roli v takových projektech, které často představují mutační analýzy stovek až tisíců vzorků, je cena fluorescenčně značených primerů. V této práci představujeme jednoduchou metodu detekce neznačených DNA fragmentů pomocí interkalace bez nutnosti přidávání interkalačního činidla do separačního gelu. Interkalátor je pouze přidán do anodového zásobníku a díky jeho migraci v opačném směru než je směr CE separace dojde ke smíchání se separovanými DNA fragmenty. Na dvou neběžněji používaných interkalačních činidlech (ethidium bromid a SYBR Green II) jsme ukázali, že tato metoda je použitelná pro rutinní detekci a kvantifikaci PCR fragmentů.

Analýza genetických změn v oblastech 17p13 a 9p21 podporuje převládající teorii o monoklonálním původu multifokálního a rekurentního karcinomu močového měchýře

Trkova M, Babjuk M, Duskova J, Benesova-Minarikova L, Soukup V, Mares J, Minarik M, Sedlacek Z.

Cancer Lett. 2006 Oct 8;242(1):68-76

Použitím analýzy mutací genu TP53 a LOH v oblastech 17p13 a 9p21 byla testována klonalita 86 tumorů získaných z 25 pacientů s rekurentním a multifokálním povrchovým karcinomem močového měchýře z přechodných buněk (TCCs). Tumory získané z většiny jedinců vykazovaly absenci nebo přítomnost stejné mutace v genu TP53 a/nebo identický LOH profil se stejnou alelickou ztrátou ve všech tumorech. Pouze dva páry tumorů ze dvou pacientů měly rozdílné nálezy, které byly v rozporu s monoklonalitou. Na základě těchto výsledků naše studie spíše podporuje monoklonální model vývoje vysoce rekurentního povrchového karcinomu močového měchýře z přechodných buněk.

Paralelní optimalizace a genotypování sady jednonukleotidových polymorfismů pomocí metody sloučení vzorků a kapilární elektroforézy v cyklujícím gradientu

Minarik M, Benesova L, Fantova L, Horacek J, Heracek J, Loukola A.

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Vzrůstající důležitost jednonukleotidových polymorfismů (SNPs) při odhadu náchylnosti k určitému onemocnění nebo predikci odpovědi na léčbu vede mnoho molekulárně diagnostických laboratoří k vývoji jednoduchých a levných metod pro genotypování SNPs. V předchozích pracech jsme představili techniku detekce mutací založenou na analýze homo- a heteroduplexních PCR fragmentů separovaných pomocí kapilární elektroforézy v cyklujícím gradientu na běžném multikapilárním sekvenátoru. Tato technika má výhodu především v tom, že je jednoduchá a nevyžaduje přečištění vzorků před jejich analýzou. V této práci ukazujeme praktické využití technologie pro genotypování SNP markerů na dvou klinických projektech zahrnujících screening 44 markerů u více než 500 pacientů. Nejprve byl pro každý SNP marker proveden návrh primerů a PCR podmínek. Poté byly na směsi 20 DNA vzorků, kde byla pravděpodobnost zachycení obou alel vyšší než u jednoho vzorku, zoptimalizovány CE podmínky nalezením správné cyklující teploty. Nakonec byl za použití opakovaného dávkování v jedné analýze, které výrazně zvýšilo počet analyzovaných vzorků, proveden screening markerů u všech pacientů. Úspěšnost optimalizace experimentálních podmínek bez jakékoli selekce SNP markerů na základě jejich sekvence nebo denaturačních vlastností byla 80% z jejich původního počtu. Následnou studií markerů s neúspěšnou optimalizací jsme se pokusili určit klíčové faktory pro úspěšnou analýzu. Popisovaná technika je vhodná pro projekty zahrnující genotypování nízkého až středního počtu SNP markerů, které bývají časté zejména ve farmakogenomických výzkumech nebo klinické diagnostice. Hlavními výhodami jsou nízká cena a jednoduché provedení a validace SNP markerů.

Frekvence výskytu vrozených DNA polymorfismů genů syntetické dráhy testosteronu v České populaci pacientů s karcinomem prostaty

Benešová, L., Fantová, L., Heráček, J., Urban, M., Sachová, J., Minárik, M.

Česká Urologie (přijato k tisku)

Cíl: Růst prostatických buněk závisí na hladině aktivního testosteronu, který je tak již v nízkých dávkách významným tumor promotorem. Sledování aktivity genů zapojených do regulace syntézy testosteronu má proto význam pro výzkum mechanismu vzniku a proliferace nádorových buněk prostaty. Hlavním záměrem prezentované studie bylo zjištění výskytu a frekvencí nejvýznamnějších jednonukleotidových polymorfismů skupiny genů syntetické dráhy testosteronu v české populaci pacientů s karcinomem prostaty.

Metodika: Do studie bylo zařazeno celkem 237 pacientů s karcinomem prostaty a 229 kontrolních osob s klinicky ověřenou diagnózou benigní hyperplazie. U každé osoby bylo provedeno vyšetření sady 30 jednonukleotidových polymorfismů z DNA získané z periferních lymfocytů.

Výsledky: Získané frekvence výskytu polymorfních alel se mezi skupinou pacientů s karcinomem a pacientů s benigní hyperplazií u většiny markerů výrazně neliší. Nadějným výsledkem je však identifikace markeru SR-49B (dbSNP ID rs4952219) v genu SRD5A2 u kterého byl zjištěn statisticky významný rozdíl poměrů jednotlivých alel i jednotlivých genotypů při srovnání sledované a kontrolní skupiny.

Diskuse: Polymorfismus SR-49B má potenciál pro odhad vrozené predispozice výskytu karcinomu prostaty. Žádný s ostatních sledovaných markerů takovou asociaci nevykazuje.

Genetické změny v genu pro receptor epidermálního růstového faktoru (EGFR) a jejich role v predikci odpovědi na léčbu nemalobuněčného karcinomu plic gefitinibem

Pesek M., Benesova-Minarikova L., Jelinkova M., Bruha F., Mukensnabl P., Minarik M.

The Lancet Oncology (zasláno k recenzi)

Cíl: Somatické mutace v tyrozin-kinázové doméně genu EGFR se ukazují být významným prediktorem klinické odpovědi na léčbu gefitinibem u pacientů s nemalobuněčným karcinomem plic (NSCLC). Cílem této práce bylo zjistit typy a frekvence EGFR mutací v české populaci pacientů s adenokarcinomem nebo bronchoalveolárním karcinomem (BAC) plic, kteří odpovídali na léčbu gefitinibem.

Materiál a metody: Do studie byli zahrnuti pouze ti pacienti, u kterých byl diagnostikován adenokarcinom nebo BAC, byli léčeni gefitinibem minimálně šest měsíců a vykazovali jasnou regresi nebo stabilizaci onemocnění. Somatické mutace byly vyšetřovány v parafínových bločcích nebo cytologických sklíčkách sekvenováním čtyř exonů (18 - 21), které kódují tyrozin-kinázovou doménu genu EGFR.

Výsledky: Na základě vybraných kritérií bylo vyšetřeno celkem 23 pacientů, 12 mužů a 11 žen, ve věku 34 – 77 let s průměrným věkem 65 let. Mutace v EGFR genu byly detekovány u 13 pacientů, 8 pacientů nevykazovalo žádnou detekovatelnou EGFR mutaci a vzorky se zbývajících dvou pacientů se nepodařilo zanalyzovat díky nízkému výtěžku izolace DNA. Nejčastějším typem mutace byla delece v exonu 19 (13 pacientů), která byla nalezena také v kombinaci s jinou mutací. Nejméně časté byly bodové mutace v exonu 18 (2 pacienti) a v exonu 20 (1 pacient). Medián přežití u pacientů s mutací byl 440 dní, bez mutace 326 dní. Ve vztahu k původní skupině pacientů s adenokarcinomem (s odpovědí a bez odpovědi na léčbu), byly mutace nalezeny u 8 z 39 žen (20%) a u 5 z 50 mužů (10%).

Závěr: Somatické mutace v genu EGFR hrají významnou roli při predikci úspěšné cílené biologické léčby. Nejčastějším typem je delece v exonu 19. Největší profit z léčby mají ženy s adenokarcinomem nesoucí mutaci v EGFR genu. U ostatních pacientů bude pro volbu vhodné léčby potřeba vyšetření dalších prognostických markerů.

Zhodnocení klinického přínosu vyšetření mutací v genech K-ras, p16 a p53 a alelických ztrát 9p a 18q ve vzorcích získaných EUS-naváděnou aspirační tenkojehlovou biopsií u pacientů s chronickou pankreatitidou a karcinomem pankreatu

Salek C, Benesova L, Zavoral M, Nosek V, Kasperova L, Ryska M, Strnad R, Traboulsi E, Minarik M

World Journal of Gastroenterology (zasláno k recenzi)

Cíl: EUS-naváděná aspirační tenkojehlová biopsie (FNA) je standardní diagnostická procedura př vyšetření podezřelé pankreatické tkáně. Somatické mutace v DNA izolované z takto získaných vzorků jsou již nějakou dobu studovány jako potenciální molekulární markery malignity. V této práci jsme analyzovali některé z nejčastějších genetických změn vyskytujících se v pankreatických neoplaziích u pacientů podstupujících FNA, s cílem zjistit jejich možné použití při rutinním diagnostickém vyšetření. Kromě běžné FNA cytologie jsme se pokusili také stanovit optimální kombinaci molekulárních markerů, která by vedla k maximální diagnostické senzitivitě a specificitě.

Metody: EUS-naváděná FNA byla provedena u 101 pacientů (63 mužů, 38 žen, 60 ± 12 let; 81 s následně diagnostikovaným karcinomem pankreatu, 20 s chronickou pankreatitidou) s fokální pankreatickou hmotou. Vzorky byly nejdříve vyhodnocovány H&E barvením následovaným detailnějším vyšetřením za použití barvení Giemsou. DNA byla izolována z buněk barvených Giemsou selektovaných pomocí laserové mikrodisekce a přítomnost mutací v genu K-ras, p53 a p16 byla následně testována použitím technik CGCE a SSCP. Dále byly zjišťovány alelické ztráty tumor supresorových genů p16 a DPC4 analýzou ztráty heterozygozity (LOH) v oblastech 9p a 18q.

Výsledky: Senzitivita a specificita u vzorků získaných EUS-naváděnou FNA byly 100% a 74%. U zbývajících 26% vzorků byly výsledky označeny jako nekonkluzivní. Vyšetření molekulárních markerů vykazovalo senzitivitu a specificitu 70% a 100% pro K-ras mutace, 24% a 90% pro p53 mutace, 13% a 100% pro p16 mutace, 85% a 64% pro alelické ztráty 9p a 78% a 57% pro alelické ztráty 18q. Pokud byly testovány různé kombinace

molekulárních markerů, nejlepší výsledky byly dosaženy u kombinace K-ras + LOH 9p (92% a 64%), K-ras + LOH 18q (92% a 57%) a K-ras + LOH 9q + LOH 18q (96% a 43%). Jestliže byly molekulární markery použity jako komplement k FNA cytologii pro vyšetření pouze nekonkluzivních vzorků, celková senzitivita detekce karcinomu byla 100% u všech pacientů zařazených do studie.

Závěr: EUS-naváděná FNA kombinovaná s analýzou K-ras mutací a alelických ztrát tumor supresorových genů p16 a DPC4 představuje velmi citlivou metodu pro detekci pankreatické malignity. Molekulární markery mohou najít své uplatnění především v tších případech, kdy jsou výsledky FNA cytologie nekonkluzivní.

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Cycling gradient capillary electrophoresis: A low-cost tool for high-throughput analysis of genetic variations

In the present work, we introduce a new type of DNA variation detection. This method represents a transfer of melting gel technique onto multicapillary electrophoresis DNA sequencing instrument with further improvements to achieve maximum sample throughput while maintaining a high performance. The main improvement comes from application of cycling (revolving) temporal temperature gradient in place of a single-sweep gradient, commonly used in similar gel-based techniques. This improvement enables utilization of multiple-injection technique, in which multiple samples are injected into the same capillary (or sets of capillaries) separated by predefined time intervals of partial electrophoresis. The periodic oscillation of the temperature results in identical separation conditions of all samples injected in such series. Using this novel approach, we demonstrate a dramatic increase in separation throughput by turning a standard commercial 96-capillary array instrument into a semicontinuous flow mutation detection system capable to screen over 15 000 samples in 24 h of operation on a single 96-capillary commercial instrument. This represents a 10-fold increase in sample throughput over the current comparable technology.

Keywords: Denaturing gradient gel electrophoresis / High throughput / Mutation / Single-nucleotide polymorphism / Temperature gradient capillary electrophoresis DOI 10.1002/elps.200305384

1 Introduction

Current effort in discovery and screening of DNA variations (point mutations and single-nucleotide polymorphisms, SNPs) is expected to yield valuable information on potential genetic risk factors as well as information on genetic variants that may lead to enhanced susceptibility for certain diseases. It is expected that discovery and massive screening of DNA polymorphisms will become essential for taylor-made drugs as well as disease gene association studies. Among the various experimental techniques for detection of DNA variations, DNA sequencing is the most universal approach [1]. Although sequencing has been the "golden standard" for a number of years, a variety of alternative approaches for detection and screening has emerged, overcoming the high cost and occasional lack of sensitivity. These methods can rely on hybridization [2], allele-specific enzymatic reactions such as PCR [3], minisequencing [4], strand displacement [5], and/or cleavage [6] or on differences in physicochemical properties of the DNA variants such as the melting equilibrium [7], or affinity towards stationary phase [8]. The readout can be performed either directly from the reaction mixture using plate readers [9], or following a separation by capillary electrophoresis (CE), [4, 10], HPLC [8] or mass spectrometry [11]. The techniques employing CE, HPLC or mass spectrometry offer an additional means of identification of the variation from a characteristic pattern of migration, elution or mass spectra. With many of the above-mentioned screening techniques, the sample throughput can be increased by processing multiple samples in parallel. This feature is essential considering the vast amount of potential sequence targets to be scrutinized.

Electrophoresis has traditionally been used for separation of DNA fragments, DNA sequencing as well as general fragment analysis [12]. A large family of slab-gel techniques dedicated to detection of DNA variations is based on differential melting of wild-type and mutant DNA fragments translated into an observable retention difference through electrophoretic sieving [13]. The original technique utilizing this principle is denaturing gradient gel electrophoresis (DGGE) in which amplified fragments of wild-type and mutant sequences are resolved during their migration in a slab gel containing a gradient of chemical denaturant. DGGE is well established in clinical diagnostics due to its relatively simplicity and ability to resolve

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Abbreviations: **APC**, Adenomatous polyposis coli; **BRCA2**, breast cancer-2; **CDCE**, constant denaturing capillary electrophoresis; **CGCE**, cycling temperature gradient capillary electrophoresis; **FAM**, carboxyfluorescein; **LTA**, lymphotoxin alpha; **SNP**, single-nucleotide polymorphism; **TMR**, tetramethylrhodamine

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close to 100% of mutations in a given target sequence [14, 15]. Following DGGE, other variants of slab-gel mutant separation methods were developed including temporal temperature gradient gel electrophoresis (TTGE) [16], in which the temperature is changing during separation and constant denaturing gel electrophoresis (CDGE), where the separation takes place at predetermined constant denaturing conditions [17]. In addition to the electrophoretic methods, a new approach based on denaturing HPLC (dHPLC) was presented [18]. dHPLC uses an ion-pair chromatography separation principle combined with precise control of the column temperature and optimized mobile phase gradient for separation of mutant heteroduplexes. dHPLC became recently very popular, since it can be easily automated and offers an option to collect the isolated heteroduplexes for further identification or confirmation by sequencing. Over the past several years, many papers describing novel mutations and SNPs found by either DGGE or dHPLC have been published [13, 18]. The main potential of DGGE and dHPLC is mostly in discovery of novel mutations rather than screening due to their relatively low throughput of approximately 5 min per analysis.

The transition in DNA separations from traditional slab-gel electrophoresis to CE systems started in early 1990 and was later further accelerated by the Human Genome Project [19]. The first use of CE for separation of DNA mutants was reported in 1994 [20]. The separation of heteroduplexes was achieved at different temperatures controlled by means of Joule heating through adjustment of the separation voltage. In 1994, a method referred to as constant denaturing capillary electrophoresis CDCE was introduced as a capillary analog to CDGE, a constant denaturing electrophoresis performed on slab gels [21]. In CDCE, the separation was carried out at an accurately maintained constant temperature at which the homo- and heteroduplex forms of wild-type and mutant sequences exhibited the best separation. In 1999, an alternative approach was demonstrated overcoming the requirement of a very accurate temperature control by applying a simple temporal temperature gradient [22]. A similar approach was later applied on a home-built capillary array instrument and recently also shown of commercial capillary array instruments [23, 24]. It was demonstrated that applying a temperature gradient, rather than a constant temperature, is useful especially in multicapillary systems, where maintaining accurate temperature across all capillaries is difficult. With a temperature gradient, each capillary reaches its temperature optimum, even if there is a difference in absolute temperature values among capillaries. The duration of such optimum separation conditions only depends on the overall slope of the temperature gradient. The detail description of temperature gradient optimization and its influence on separation selectivity and resolution is described elsewhere (Bjørheim *et al.*, submitted). The temperature gradient approach is very promising for detection of DNA variants, since it can be applied to many existing commercial multicapillary CE systems with no additional requirements on instrument hardware. The typical run time is less than 60 min, depending on the resolution requirement (*i.e.*, gradient slope). With an automated 96-array capillary array instrument this results in a sample throughput of 1536 samples in 24 h [25]. It is clear that in order to apply this technology for massive screening of mutations or SNPs, the sample throughput needs to be further increased.

In the present work, we introduce a new type of DNA variation (mutation or SNP) screening technology based on capillary array electrophoresis. The PCR amplified fragments of known sequence variants are detected based on their differential migration in a polymer-filled capillary. We are using cycling (oscillating) temporal temperature gradient to compensate for local fluctuations in temperatures across the multicapillary array. In addition, the application of short periodic temperature cycles allows us to continue injecting subsequent sample plates between the temperature gradient cycles before the earlier samples appear in the detector. Using this novel approach, we demonstrate a dramatic increase in separation throughput by more effective utilization of the separation capacity (volume) of each capillary.

2 Materials and methods

2.1 Chemicals

All experiments were performed using standard MegaBACE™ buffers and MegaBACE™ LPA long-read matrix (Amersham Biosciences, Piscataway, NJ, USA). PCR primers were obtained from MedProbe (Oslo, Norway). The primer with GC clamp were labeled with 6-carboxyfluorescein (6-FAM) on the tested samples and tetramethylrhodamine (TMR) on the internal standards. Primer used for SNP in the lymphotoxin alpha (LTA) gene (accession number 153440, locus 6p21.3, NCBI reference SNP ID: rs 909253), 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCT GGT GGG TTT GGT TTT GG 3' and 5' GAG CAG AGG GAG ACA GAG AGA G 3'. Primer used for mutation in Adenomatous polyposis coli (APC) gene (accession number 175100, locus 5q21-q22, 1p34.3-1p32.1, exon 15, codon 1450), 5'- CGG GCG GGG GCG GCG GGA CGG GCG CGG GGC GCG GCG GGC GAG CAT TTA CTG CAG CTT GCT 3' and 5' ACC TCC TCA AAC AGC TCA AA 3'. Primer used for SNP in the breast cancer-2 (BRCA2) gene (accession number

600185, locus 13q12.3, NCBI reference SNP ID: rs573014), 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GAA GG TAT GTG CAT TGT TTT T 3'and 5' CCG CAA TAA AGC AAA TAT TAC 3'.

2.2 CE

All CE experiments were performed on an MegaBACE 1000 96-capillary DNA analysis system (Amersham Biosciences). The instrument was equipped with an automated loading robot Caddy 1000 (Watrex Praha, Prague, Czech Republic), to allow for unattended automated operation. To reach high temperatures needed for some mutant separation, the temperature sensor was equipped with an additional resistor resulting in a positive offset of temperatures by approximately 10°C. The temperature was recorded using Fluke logging thermometer with FlukeView software (Michell Instrument, San Marcos, CA, USA). The temperature profiles were constructed using MegaBACE configuration selector (MBCS) software (Genomac International, Prague, Czech Republic). The data was processed by SNP Profiler™ software (Amersham Biosciences).

2.3 PCR reaction

Full blood from blood donors at Ulleval Hospital (Oslo, Norway) was anonymously collected and genomic DNA was extracted with QIAamp DNA Blood Mini Kit from Qiagen (Valencia, CA, USA). All reactions were performed on a PTC-200 thermal cycler (MJ Research, Waltham, MA, USA), by mixing 50 ng of genomic DNA with 25 mM of each dNTP (Abgene, Epsom, UK), 10 × *Taq* buffer, 1 unit of *Taq* polymerase (Applied Biosystems, Foster City, CA, USA) and 5 pmol of each primer (MedProbe) in a final volume of 25 μL. Same cycling conditions were applied for amplification of all fragments. The cycling program included denaturation for 60 s at 94°C, annealing for 60 s at 53°C and elongation 60 s at 72°C, for 35 cycles. Where applicable, heteroduplexes were formed by heating the PCR products at 94°C for 5 min, then annealing the fragments at 65°C for 60 min followed by slow cooling to 4°C.

2.4 Optimization of cycling temperature range

For each target sequence, extended by the GC clamp, the theoretical melting temperature profile was first calculated using WinMelt™ simulation program (Medprobe) based on Poland's algorithm [26]. A melt profile will show regions of theoretical high and low melting domains of a known sequence. Location of primers and GC clamps can be optimized by analyzing their effect on the overall fragment melting profile. The temperature oscillation was within ± 1°C range from the melting temperature of the low-melting domain (target sequence).

3 Results and discussion

The main purpose of the present work is to extend current technology for detection of DNA variants into a high-throughput mode. Previously, we have described screening of multiple samples for the presence of somatic mutations after transferring a high-resolution technique of CDCE from single-capillary format to a commercial capillary array instrument [27]. Later we have demonstrated additional advantages of applying a temporal temperature gradient rather than maintaining an accurate constant temperature for increased reproducibility in a commercial multicapillary instrument [23]. The present paper describes the next direction in the effort to further increase the sample throughput by means of utilizing recently introduced multiple-injection technology [28].

In a multiple-injection experiment, samples are serially injected into the capillary (or array of capillaries) in periodical time intervals separated by short application of separation voltage [29]. The main advantage of this approach is more efficient usage of the separation capacity (migration volume) of the capillary column. Following injection of the first sample (or a set of samples in capillary array), a so-called interval voltage is applied for a sufficient period of time (typically 2–5 min for short oligonucleotides) preventing overlap of the slowest peak from the sample with the fastest migrating peak from the next sample. After this period, the next sample is injected resulting in a continuing process during which the first set of samples is reaching detectors, the following set is separating and new ones are being injected. In the most common version, the injections are repeated several times before the fastest migrating peaks from the first sample reach the detector. After the final injection, a run voltage is applied for a longer period of time (30–60 min) to drive all peaks from all injections pass the detection window. The maximum number of injections must be determined experimentally. Indeed, the multiple-injection technique is only applicable to samples containing compounds with relatively narrow windows to prevent overlap of bands from different injections (paper in preparation). For separation of wild-type and mutant fragments based on differential melting, this condition applies. In most of these cases, the time difference between the fastest migrating peak from the mixture (usually the unincorporated primer) and the slowest migrating peak (usually the most denatured fragment) is typically around 10 min relative to the total analysis time of 30–40 min [7, 24]. The

multiple-injection scheme could be directly applied to DNA separation, where samples would be serially injected and separated at a constant temperature (Fig. 1A).

A different situation occurs if a temperature gradient needs to be applied. In temperature gradient capillary electrophoresis (TGCE), the running temperature is gradually changed during the entire run. The fundamental assumption is that the samples have to be subjected to a proximity of optimum melting conditions over a sufficient duration of their migration in the capillary. With multiple injections, the samples eluting at the beginning would be subjected to different temperature range compared to the ones injected later as shown in Fig. 1B. In order to subject samples from all injections to identical melting conditions, the temperature has to be periodically changed to follow the profile of repeatedly injected samples. With this arrangement, each sample undergoes the same number of temperature gradient cycles and thus is subjected to the same melting conditions (Fig. 1C).

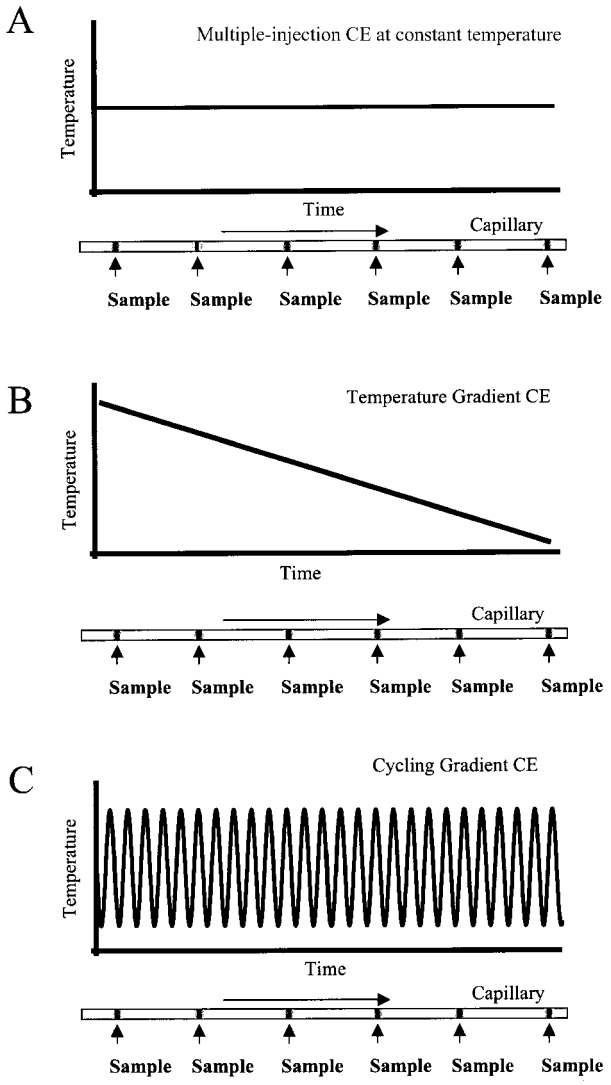

Figure 1. Scheme of (A) multiple-injection CE, (B) multiple-injection TGCE and (C) multiple-injection CGCE. The upper drawing illustrates the temperature profile. The simulated positions of separated sample zones are in the bottom.

In a previously demonstrated experiment, samples (PCR products) containing an artificial high-melting domain (CG clamp) were subjected to a temperature gradient to cover a range of optimum melting conditions [7]. A typical result of this single-sweep gradient experiment (TGCE) is shown in Fig. 2A. Here, an LTA mutant was subjected to a descending temperature gradient starting at 52°C and ending at 48°C with a rate of 0.1°C per min. During the gradient, a period of time at which the samples are subjected to their optimum melting conditions is given by the gradient slope and has direct impact on separation resolution [30]. This period could be subsidized by a series of cycles during which the separated samples are several times subjected to the melting optimum. Figure 2B shows a separation of the same sample (LTA) subjected to a cycling temperature gradient. It can be seen that the resolution in this case is fully comparable to the single-sweep gradient experiment from Fig. 2A. It seems that the overall retardation effect due to partial denaturation in case of a cycling gradient is similar to a single-sweep gradient.

Most commercial capillary array instruments allow controlling temperature during the run. Figure 3 shows the temperature profile of a single-sweep gradient (A) and a

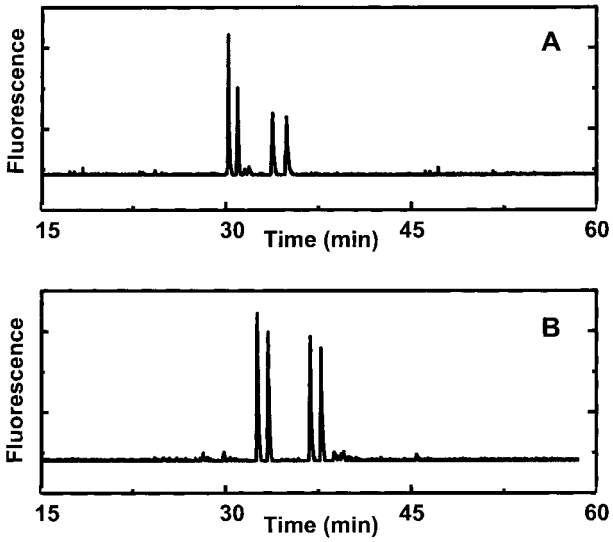

Figure 2. Comparison of two temperature profile modes. Separation of LTA variants (A) in a single-sweep gradient mode and (B) using a cycling temperature gradient mode. The peak resolution is fully maintained when a cycling gradient is applied.

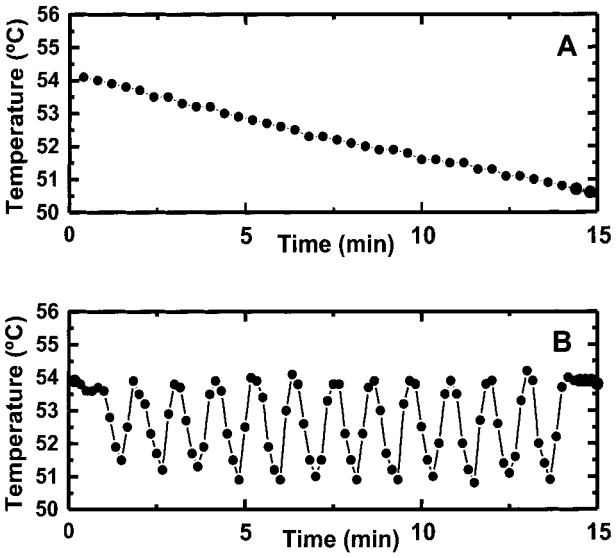

Figure 3. Experimental measurements of temperature profiles inside MegaBACE 1000 capillary chamber. Typical profile of a (A) single-sweep gradient mode and (B) a cycling temperature gradient mode. The instrument was equipped with high-temperature setting adaptor.

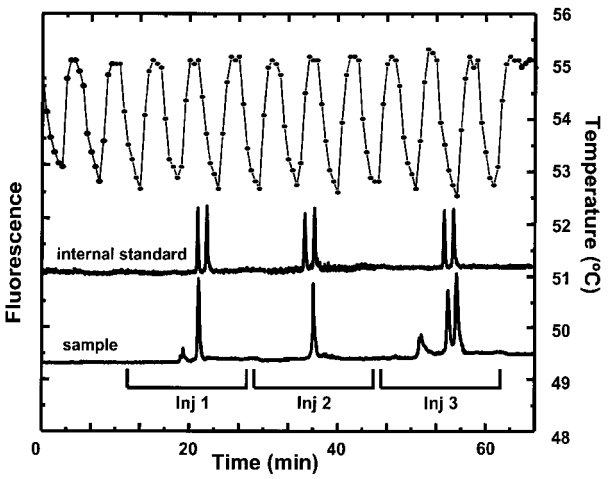

Figure 4. Analysis of several samples for APC mutation using multiple-injections and fast cycling temperature gradient conditions. The three injections were performed every fourth temperature cycle. First injection is C homozygote, second injection is T homozygote and the last injection is C/T heterozygote.

typical cycling gradient (B) recorded inside the capillary chamber of MegaBACE 1000 capillary array instrument equipped with high temperature setting. Investigation of further aspects of temperature cycling as well as generalized optimization of cycling parameters will be described separately (Bjørheim *et al.*, submitted). The unchanged separation performance of the cycling gradient compared to a single-sweep gradient allows to apply multiple-injection method. Depending on the actual application, the samples can be periodically injected either in every gradient cycle or once in every couple of cycles. An example of multiple-injection cycling temperature gradient capillary electrophoresis (CGCE) with fast temperature cycling (5 min per cycle) is shown in Fig. 4. The APC mutant samples were injected in every fourth gradient cycle intervals. During each cycle, a temperature gradient going from 55°C to 53°C and back to 55°C was applied. An internal standard containing 1:1 mixture of individually amplified wild-type and mutant fragments labeled with a TMR dye was included in each sample well. Since no heteroduplexes were formed following the PCR, the resulting separation patterns consists only of homoduplexes. It can be seen that a full separation of both, wild-type and mutant homoduplexes of the internal standard was achieved within the individual interval windows. Clearly, a very high resolution of the peak separation is achieved under the cycling temperature gradient conditions, allowing identification of both alleles in a heterozygous sample by comparison to the peak pattern of the standard. Apart from just detecting a presence of DNA variation from the characteristic peak pattern in heterozygous samples, the complete separation of the two homoduplexes allows direct identification of homozygous genotypes. This is the key in automated SNP genotyping, where the two homozygous genotypes can be directly scored. An example of high-throughput analysis and automated scoring of SNPs is shown in Fig. 5. There, a BRCA2 SNP was screened in various patients using the five injections with 45°C–43°C cycling temperature gradient. Each sample included a 6-carboxy-*N,N,N',N'*-tetramethylrhodamine (TAMRA)-labeled internal standard. The data was processed using SNP Profiler software which allows assignment of individual injection windows. The genotypes are directly determined from the positions of mutant homoduplex peaks coeluting with the internal standard homoduplex peaks.

The total runtime of the experiment shown in Fig. 5 was less than 2 h. We estimate that on a common 96-capillary instrument five injections could feasibly be performed without any adverse effect on the separation matrix. The total runtime would then be 20 min of initial "dead" volume + 5 × 15 min separation window + 15 min final electrophoresis = 110 min. Considering 10 min additional periods required for gel replacement in between runs, a total of 12 of similar multi-injection runs can be performed in less than 24 h of operation. This represents an overall throughput of 5760 samples in 24 h. Commercial genetic analyzers are usually equipped with four dye channels. Considering that up to three fluorescent dye channels

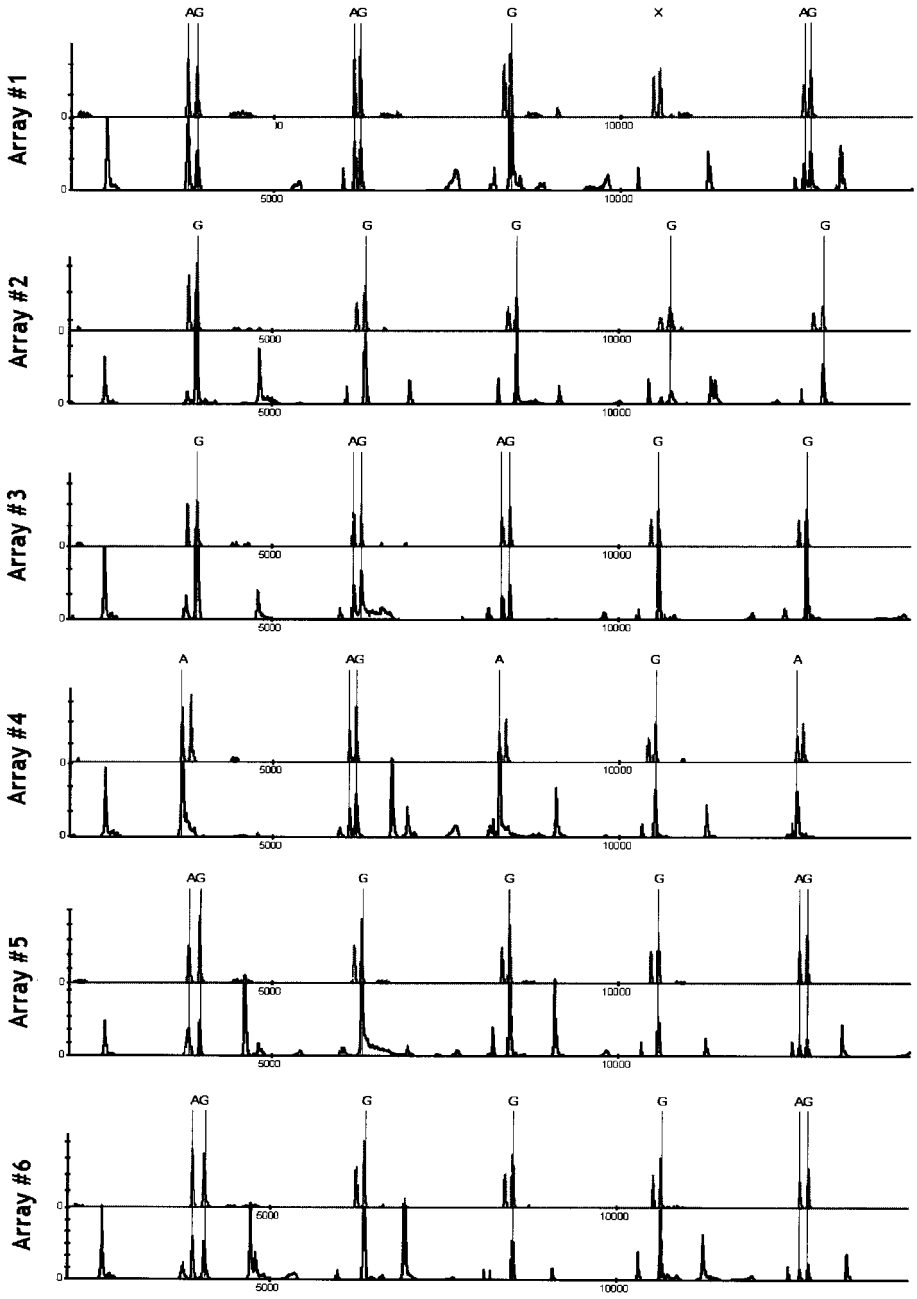

Figure 5. Illustration of high-throughput SNP scoring by CGCE in multicapillary format. Five different sample plates (FAM-labeled PCR fragments of BRCA2) were consecutively injected during a cycling temperature gradient (see Section 3 for details). Allele scoring was performed against a TMR-labeled internal mutant showing heterozygote profile with both allele peaks. The internal standard was also used as an "injection marker" for consecutive data processing by SNP Profiler software.

can be used to detect unknown samples (the last channel is assigned for the internal standard), it is clear that the potential capacity can be further increased 3-fold up to 17 200 samples in 24 h on a single 96-capillary instrument. Unlike in other mutant or SNP scoring methods, the presented technology includes a very straightforward workflow shown in Fig. 6. Following the original PCR amplification, there is no sample cleanup required. Using a 96-format, PCR cycler enables complete automation from sample preparation to the multiple-injection CE analysis using robotic plate handlers.

4 Concluding remarks

In the present work, we demonstrated that the general principle of applying a temporal temperature gradient in CE could further be extended into applying a periodical temperature cycle. As in similar techniques described before, in CGCE we use PCR with one of the primers extended by a high-melting domain to amplify a target DNA sequence surrounding the mutant or SNP marker. The PCR conditions are specific for each target sequence.

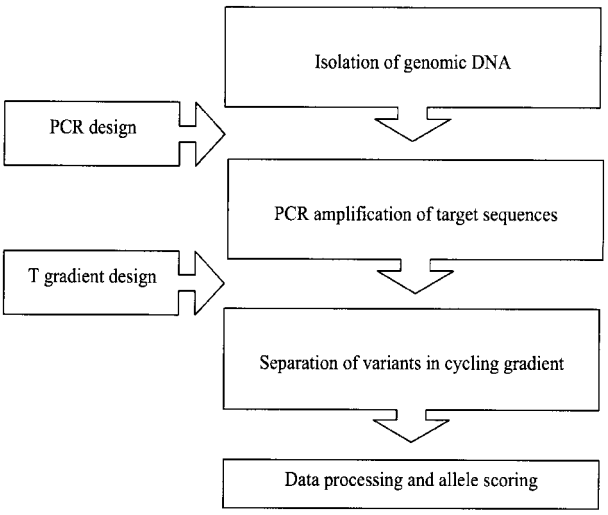

Figure 6. An overview of CGCE sample processing workflow.

The application of periodical cycles allows a better compensation of the local temperature fluctuations inside the multicapillary oven. Rapid gradient cycling with rates of up to several cycles per minute showed better results in comparison with slower cycling intervals. In addition, the instrument hardware does not appear to be able to follow the rapid temperature changes inside the chamber resulting in a relative constant average temperature of the optical components. Periodical application of the temperature gradient also enables usage of multiple injection technology, in which different samples are serially injected between the cycles and separated under the same revolving temperature conditions. Multiple-injections allow for significant increase in sample throughput. Considering the ease of sample preparation, PCR directly followed electrophoresis without any post-PCR treatment such as desalting or removal of unincorporated primers. Evaluation of the mutant presence or SNP genotype is done solely based on an internal standard running in a separate spectral channel. In situations where a slower migrating PCR fragment would directly overlap with a faster migrating primer peak from a subsequent injection, the spacing between the injections needs to be adjusted. For a given mutant/SNP marker, the peak distance is very reproducible, therefore the injection spacing can easily be optimized accordingly. This technique represents a cost effective, simple and powerful tool for high-throughput scoring of DNA mutants and SNPs.

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Application of cycling gradient capillary electrophoresis to detection of APC, K-ras, and DCC point mutations in patients with sporadic colorectal tumors

A previously introduced technique of cycling gradient capillary electrophoresis (CGCE) was applied to monitoring of molecular changes during adenoma-carcinoma transition in progression of sporadic colorectal cancer. The purpose of this work was optimization of separation parameters for selected mutation regions in tumor suppressor genes involved in the early stages of colorectal carcinogenesis, followed by scanning for these mutations in clinical tissue samples from patients with adenomatous polyps and early carcinomas. A total of 47 colorectal tumors in various stages of progression were examined. Main emphasis was given to evaluation of mutation detection sensitivity and specificity required for effective early disease detection. A total of 7 different somatic mutations was identified among 32 K-ras mutant samples, 1 inherited mutation and 5 somatic mutations were identified among 15 adenomatous polyposis coli (APC) mutated samples. None of the two previously reported "deleted in colorectal carcinomas" (DCC) mutations was found in any of the clinical samples. In addition to simple optimization of running conditions, CGCE has demonstrated sensitivity and selectivity allowing detecting small mutant fractions as well as combination of multiple mutants within a single target sequence.

Keywords: Adenomatous polyposis coli / Colorectal cancer / Cycling gradient capillary electrophoresis / K-ras / Mutations
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1 Introduction

Colorectal cancer represents a leading cause of cancer-related death in developed countries (second overall after cardiovascular diseases). Current studies positively show increasing incidence as well as mortality rates, which can be attributed to exposures to various factors including high fat diet, smoking, environmental carcinogens, *etc.* [1, 2]. A number of reports on colorectal carcinogenesis suggest a model based on deactivation of various tumor suppressor genes during adenoma-carcinoma progression [3, 4]. According to this model, early stages of tumor initiation and growth are indicated by accumulation of somatic DNA mutations in several tumor suppressor genes, followed by loss of one or both alleles [5]. A scheme of molecular changes during the colorectal carcinogenesis is shown in Fig. 1. The initiation of tissue proliferation towards adenoma is often accompanied by mutations within mutation cluster region of exon 15 in the in adenomatous polyposis coli (APC) gene. The region extends over 200 codons harboring over 500 mutations currently registered, with the most frequent mutations around codon 1300 and 1450 [6]. As illustrated in Fig. 1, somatic mutations in APC are followed by development of small adenomas often exhibiting substitutions in mutation hotspot of K-ras (codon 12 and 13) exon 1). Further proliferation of the adenoma and its transformation into malignant carcinoma is indicated by loss of heterozygosity at locus 18q21.3 containing the DCC gene (deleted in colorectal carcinoma) [7]. Scarce reports of mutations within DCC include one substitution in exon 28 and another substitution in intron 14 [8]. Malignant tumors further often exhibit deactivation of p53 protein through a number of possible TP53 gene mutations, most of which occur in exons 5–8 [9]. The described diagram suggests usability of monitoring somatic alterations in APC and K-ras genes for detection of tumor 4 initiation and DCC and TP53 for possible estimation of malignant potential in large adenomas and developed tumors. Detecting the above mutant markers in adenomatous polyps facilitates early detection of the tumor progression

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Abbreviations: APC, adenomatous polyposis coli, **CGCE**, cycling gradient capillary electrophoresis; **DCC**, deleted in colorectal carcinomas

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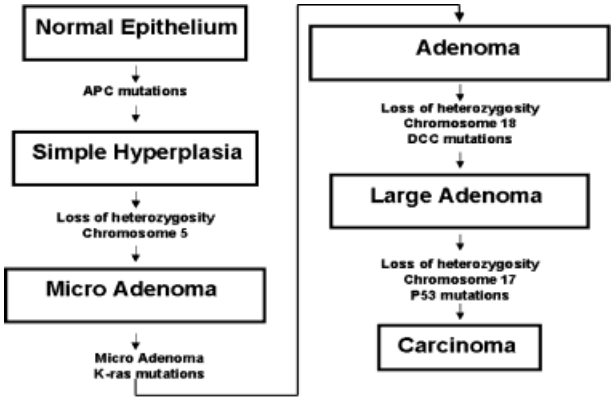

Figure 1. Simplified model of molecular genetic changes during tumor progression of colorectal cancer.

and might result in a better chance of survival. Especially, detection of low-level point mutations has a significant potential for cancer prevention through early diagnosis.

The recently introduced technique of cycling gradient capillary electrophoresis (CGCE) represented an ideal automated tool for parallel monitoring of mutants in multiple clinical samples [10]. The present work describes the optimization of mutation detection in several tumor suppressor genes involved in colorectal carcinogenesis followed by analysis of clinical samples taken from patients during colonoscopy treatment.

2 Materials and methods

2.1 Sample preparation

DNA samples were collected from large polyps ($>$ 10 mm) in colon and rectum from patients by polypectomy during colonoscopy examination. Tubular adenoma was found in most cases (70%), 5% of adenomas were tubulovillous, while none exhibited villous structures. The levels of dysplasia were mild (22.5% of cases), mild to moderate (30% of cases), moderate (12.5% of cases), and severe (10% of cases). Each polyp was dissected into smaller parts (3–5 mm) and each part was then processed separately. Genomic DNA was isolated from tissue samples using JetQuick isolation kit (Genomed, Loehne, Germany). Extracted DNA (~50 ng) was subjected to PCR amplification with fluorescently labeled primers using conditions specific to each mutant (see Table 1). Artificial mutant standards were prepared by PCR amplification of a wild-type DNA using a special extension on one of the PCR primers. The extension included the mutated base in place of the wild-type base. The following mutation regions were examined; selected mutation hotspots within the mutation cluster region of APC gene (codons 1243–1310 and 1413–1465), K-ras hotspot codon 12 and 13 of exon 1, and 2 mutation positions within exon 28 and intron 14 in DCC [8].

2.2 CGCE

After PCR amplification, mutant heteroduplexes were analyzed in periodically cycling temperature gradient on MegaBACE capillary-array genetic analyzer (Amersham Biosciences, Sunnyvale, CA, USA) equipped with a Caddy plate loading robot (Watrex Praha, Czech Republic) for unattended operation. The separation took place in a standard denaturing gel matrix (MegaBACE long-range matrix; Amersham Biosciences) containing 7 M urea. The running conditions included injection for 120 s at 3 kV and running at 6 kV for 90 min. The cycling gradient temperature profiles were created using MBCS software Version 2.0 (Genomac International, Prague, Czech Republic).

Table 1. Optimized conditions for mutation analysis in selected mutation regions

Target	Primer sequence	Theoretical melting temperature	CGCE temperature range
APC1	5'-GTTCATTATCATCTTTGTCATCAGC-3' 5'-FL-[GC]-TTTATTTCTGCTATTTGCAGGGTA-3'	71°C	51–49°C
APC2	5'-FL-[GC]-CCATGCCACCAAGCAGAAGTA-3' 5'-TCTCTTTTCAGCAGTAGGTGCTT-3'	70°C	52–50°C
K-ras	5'-ATGACTGAATATAAACTTGTG-3' 5'-FL-[GC]-CCTCTATTGTTGGATCATATTC-3'	70°C	52–50°C
DCC–C9	5'-FL-[GC]-TTTTCAACACACAATCCCTTT-3' 5'-TCATGCAAACTTACCCATTATGA-3'	65°C	45–43°C
DCC–D114	5'-TCATCACTGTGTTTTCTATTTCAGG -3' 5'-FL-[GC]-ACAGACACAGGAAGCAAA-3'	73°C	53–51°C

[GC] denotes a high-melting clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG. FL denotes labeling by fluorescein.

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The temperature ranges were optimized for each marker (target sequence). The optimization is described in Section 3. All primers and CGCE running conditions used in this work are listed in Table 1.

3 Results

The analysis of somatic point mutations shows potential for early detection of colorectal tumors through mutation analysis of bioptic tissue samples. We have previously introduced CGCE for high-throughput screening of single-nucleotide polymorphisms (SNPs) using multiple injection approach [10]. In the present work, we demonstrate the applicability of this method in clinical research by detecting several point mutations in tissue samples taken during regular colonoscopy examination. All mutation regions analyzed in this study were previously found to be related to colorectal carcinogenesis.

Prior to detecting selected mutation markers, it is necessary to optimize temperature conditions for all selected target sequences. Mutation detection techniques based on separation of homo- and heteroduplexes often require careful optimization of separation conditions including optimum melting temperature [11] in electrophoresis or temperature in combination with mobile phase gradient composition in chromatography [12]. Similarly, in a traditional single-sweep temperature gradient approach (TGCE), the gradient range as well as gradient slope has to be optimized in order to find the best conditions to resolve all forms (wild-type and mutant homoduplexes and two heteroduplexes) potentially present in the sample [13]. In CGCE, the only parameter to optimize is a range of temperatures while the individual gradient cycles are performed rapidly with no delay. It was demonstrated previously that for high reproducibility fast cycling is preferential to slower temperature sweeps [14]. The maximum achievable frequency of gradient cycling is usually limited by the rate of temperature control inside the capillary oven and is typically lower with increasing range of the cycling gradient. Optimization of a new marker usually involves performing several runs using different temperature ranges of cycling gradient. During optimization, cycling temperatures are usually selected 2°C above and below the theoretical melting temperature of the target DNA segment. An example of optimization of the temperature cycling conditions is shown in Fig. 2. The PCR product of DCC gene wild-type target sequence was mixed at a 1:1 ratio with an artificial point-mutant standard (see Section 2). The intron 14 mutant, labeled as C9, was previously found in colorectal carcinoma patients [8]. After mixing, 5 min denaturation was performed at 95°C followed by reannealing of wild-type and mutant strands at 65°C for 60 min. The resulting four combinations of

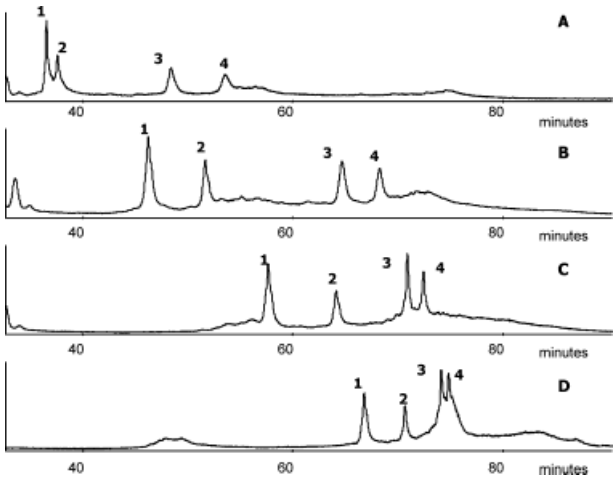

Figure 2. Optimization of CGCE running parameters. DCC intron 14 mutant standard analyzed at different ranges of cycling temperatures: (A) 43°C–41°C, (B) 44°C–42°C, (C) 46°C–44°C, (D) 48°C–46°C. An optimum temperature range of 44°C–42°C was used for mutation analysis in clinical samples. Similar optimization was performed on all mutation regions used in the study (see Table 1).

homo-and hetero- duplexes (labeled 1–4) were analyzed by CGCE at four different gradient cycling ranges. It can be seen that at lower temperature range the wild-type (1) and mutant (2) homoduplexes are not completely resolved. Clearly the best resolution was achieved when cycling between 42 and 44°C. This is in agreement with theoretical predictions, indicating natural melting temperature of the DCC intron 14 target sequence of 64°C since the 7 M urea in the separation matrix effectively lowers the DNA melting temperature by approximately 21°C [15, 16]. At a gradient higher than the optimum, the two heteroduplexes (3 and 4) co-elute. Using the identical procedure, conditions were developed for all target sequences examined in this study. The final optimum conditions for all markers are listed in Table 1. These conditions were used to examine the clinical samples.

One of the most important parameters of each mutation detection technique is detection sensitivity expressed in terms of a fraction of mutated DNA copies detectable in an excess of wild-type DNA [17]. While conventional DNA sequencing typically detects a presence of minor allele at a concentration above 20%, techniques based on spatial separation of mutant and wild-type (such as denaturant gradient gel electrophoresis, DGGE, or single-strand conformation polymosphism, SSCP) are capable of detecting low-level mutations in fractions down to 10% or lower [18]. An example of detection sensitivity using CGCE is shown in Fig. 3. Two clinical samples, positive for mutation in K-ras mutation hotspot (codon 12 and 13), showed pattern of heteroduplex peaks at the optimum separation

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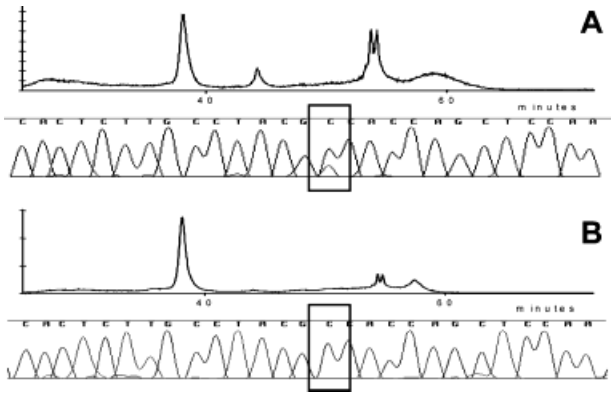

Figure 3. Illustration of CGCE sensitivity. K-ras mutant region (exon 1) was examined in two patients with different levels of mutated DNA fraction. From the high mutant fraction (A), the G→A substitution at codon 13 could clearly be identified by sequencing (complimentary strand was sequenced). If the same mutant is present in a low lever (B), it is still clearly visible in the CGCE trace but not detectable by sequencing.

temperature range of 52°C to 50°C (Figs. 3A and B). The first sample clearly contains a larger fraction of the mutated DNA compared to the second sample. While in the first case the mutations could be identified by resequencing of the PCR product (Fig. 3A, bottom), no mutations were found when sequencing the second sample containing lower fraction of the mutant (Fig. 3B, bottom). The fraction presented in Fig. 3B was estimated at ~ 10% level. This estimation was based on a separate calibration experiment in which different fractions of artificial mutant and wild-type were mixed (data not shown) and is in agreement with similar calibration experiments published previously [19].

The optimized conditions were used for analysis of the five selected mutation regions in a total of 47 tissue samples. An overview of mutants found during the clinical study is summarized in Table 2. Mutations in APC gene were found in a total of 9 samples (9/47, 19%). Out of these 9 samples, 6 different mutations were identified. An overview of the 6 different APC mutations and one double-mutant sample is shown in Fig. 4 together with a nonmutated sample used as a reference. The exact mutation positions, denoted as the codon number and the mutation type in each electropherogram window, were confirmed by sequencing. Mutation in codon 1442 (T→A substitution) was confirmed as inherited by its presence also in the blood sample taken from the same patient. The identical inherited mutation was found in tissue as well as blood samples of yet another patient. In the tissue sample, however, it was in combination with another somatic mutation at codon 1450 (C→T substitution)

Table 2. Overview of results of mutation analysis in 47 clinical tissue samples

Gene	Location		
	Ascendens (15 tumors)	Descendens (7 tumors)	Sigmoideum/ rectum (25 tumors)
APC	1 (7%)	3 (43%)	5 (20%)
K-ras	5 (33%)	3 (43%)	15 (60%)
DCC	0 (0%)	0 (0%)	0 (0%)

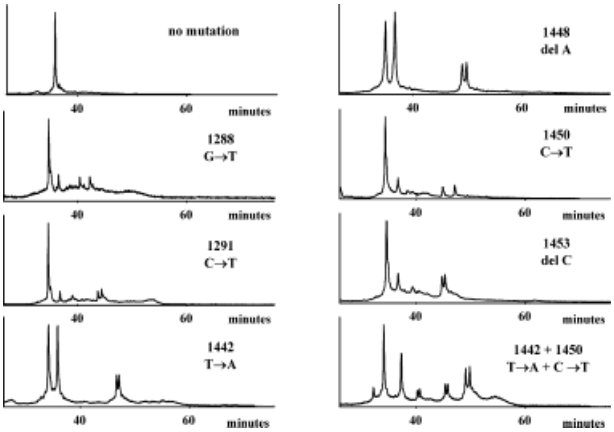

Figure 4. Overview of various types of APC mutations found during clinical CGCE of 47 tissue samples. Upper left trace, nonmutated reference. Bottom right trace, double-mutant with one inherited mutation (T→A substitution in codon 1442) and a second somatic mutation (C→T substitution in codon 1450). The inherited codon 1442 mutation was also found separately in blood of another patient (bottom left).

resulting in a double-mutant (Fig. 4, bottom right). The two concurrent mutations produce a pattern of multiple peaks representing wild-type and mutant forms with two pairs of heteroduplexes for each of the mutants plus an additional heteroduplex pair resulting from mutual combination of the two mutant sequences. The most frequently found mutations were in K-ras oncogene. Out of 47 tissue samples, 22 have exhibited a K-ras mutation. Seven different types of somatic mutations were identified from the 22 K-ras positive samples. An overview of K-ras mutations is presented in Fig. 5. It can be seen that most mutations were localized in codon 12, while only one was from codon 13. Similarly to the above APC gene, a double-mutant was found in one of the patients (Fig. 5, bottom left). While one of the mutations was identified as G→A substitution in codon 12, it was not possible to identify the second mutation, clearly detected in the CGCE electropherogram (see Section 4). None of tumors was positive for any of the two DCC mutations. When comparing

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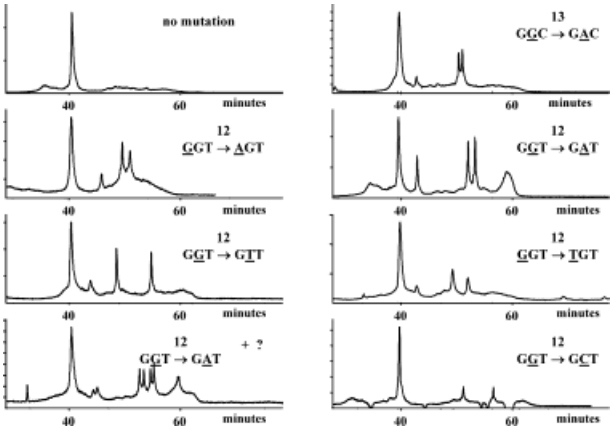

Figure 5. Overview of various types of K-ras mutations found during CGCE analysis of 47 tissue samples. Upper left trace, nonmutated reference trace. Bottom left trace, mutant identified as G→A substitution in codon 12 and unidentified mutant, presumably from the same codon (see text for details).

mutation frequency based on tumor location, we have found more mutated tumors in colon descendens (30/47, 64%) than in colon ascendens (15/47, 32%).

4 Discussion

The application of cycling gradient significantly simplifies the optimization of separation conditions in comparison to a single-sweep gradient. The optimization only involves finding the temperature range and does not require complicated matching of the gradient duration (slope) to the duration of migration of fragments in the capillary. An important feature for clinical applications is mutant detection sensitivity. Bioptic tissue samples are usually collected from areas of macroscopic changes or early adenomas, which often contain only a small portion of cells carrying mutated DNA. This requires sensitive techniques capable of detecting low levels of mutants. The presented example showed capability to unambiguously detect mutated fractions of ~10%. The maximum sensitivity reached in an unrelated experiment was 1% (data not shown). A possibility to further increase the sensitivity (lower the mutant fraction detected) is now under investigation. One potential approach is enrichment of heteroduplex forms using fraction collection followed by PCR reamplification of the isolated fractions.

Specificity of mutation detection techniques becomes crucial when more than one mutation present within the target sequence is to be detected. Such situation is common in the case of monitoring alterations in tumor suppressor genes in dysplasic colon tissue, which often exhibit multiple mutations at once. Distinguishing the different point mutations has a prognostic value, since different mutations often exhibit different impact on malignancy and survival rates [20]. In the presented example, both mutations are clearly distinguishable from the resulting combination of heteroduplex forms of individual mutants. The presence of two mutants within a single APC target sequence are typical for a heavily mutated cancer tissue. The fact that one of the mutations was inherited (found also in blood of the patient) certainly suggests the initial phases of tumor suppressor inactivation [21]. The importance of this inherited mutation, which was also found in another patient's blood sample, is now under study. Also a subject to further investigation is an unidentified second K-ras mutation from a sample shown in Fig. 5, bottom left. By mixing with a set of know mutations, it was preliminarily concluded that the mutation is probably at the exact same position as the first mutation (G → A at codon 12) but must likely involving a different base substitution. This theory will be examined in more details, however, the inability to identify the second mutation further confirms the usability of CGCE over direct sequencing.

In the presented study, 28 mutants were identified from a total of 47 tumor samples, 8 samples exhibited both APC and K-ras mutations. The overall higher frequency of K-ras-positive samples compared to APC positives can be attributed to the localized hot spot within the codons 12 and 13 of K-ras allowing virtually 100% of K-ras mutants to be detected. In addition, it may be possible to identify various K-ras mutants using an internal artificial mutant standard as an alternative to direct sequencing [22]. With APC gene, it is expected that some portion of samples with mutation outside the monitored mutation cluster region escapes the assay. In contrast with the expectations, no sample was positive for any of the two DCC mutations monitored in the study [8]. Finally, the overall higher frequency of the APC and K-ras mutants in colon descendens combined with sigmoideum and rectum (25/32, 78%) compared to colon ascendens (6/14, 43%) is in an agreement with the clinically observed higher tendency of the malignant polypoid growth in the left colon [23].

CGCE represents a viable tool for mutation detection. The technique exhibits high sensitivity and specificity, which is often required for mutation analysis in clinical samples such as bioptic or resection tissue *etc.* The method does not require any sample manipulation prior to capillary electrophoretic separation and is compatible with two commercially available capillary array sequencing instruments [24, 25]. The main advantage of the technique over currently existing TGCE analogues is in simple optimization of separation conditions and a possibility of multiplexing by multiple-injection approach [10].

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Multicapillary electrophoresis of unlabeled DNA fragments with high-sensitive laser-induced fluorescence detection by counter-current migration of intercalation dye

Analysis of PCR fragments for applications, such as screening of nucleotide polymorphisms, detection of somatic mutations, or quantification of reverse-transcription PCR products, becomes central in clinical research as well as preventive testing, diagnostic screening, and pharmacogenomic genotyping. A variety of CE techniques, utilizing great potential of multicapillary-array sequencers, is now commonly applied in prevention, diagnosis, and treatment of a wide range of genetic diseases (cancer, cardiovascular, and neurodegenerative diseases, *etc.*). Costs of fluorescently labeled primers is often a major factor in large-scale projects requiring mutation analysis in hundreds or thousands of samples. In the present paper we introduce a simple approach of detecting unlabeled DNA fragments through intercalation without a need for adding intercalator to the separation polymer matrix. The dye is only added to the anode reservoir, and mixing with the separated DNA fragments takes place upon its migration opposite to the direction of the CE separation. Using two common intercalating dyes (ethidium bromide and SYBR Green II) we present this method as a tool for routine PCR detection and quantification.

Keywords: CE / DNA / LIF / Intercalation dye / Laser-induced detection
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1 Introduction

DNA fragment analysis by CE has become the preferred instrumental technique for screening of genetic abnormalities in a variety of applications, including research and diagnosis of diseases, preventive testing, as well as genotyping for personalized medicine [1]. The main advantage of CE over previously used slab-gel electrophoretic approaches is in speed, sensitivity, resolution, and a possibility of automation, allowing to process hundreds of samples in an unattended reproducible manner. Commercial single and multicapillary CE systems are equipped with LIF detection, enabling high sensitivity for exposure of small concentrations of DNA fragments. LIF detection requires fluorescent labeling of analyzed DNA fragments, which is mostly achieved by using fluorescently labeled primers in PCR amplification reaction preceding the CE analysis. Depending on the type of the fluorescent dye and the length of the oligonucleotide sequence, a labeled primer may be up to tenfold more costly when compared to its unlabeled form [2]. This additional expense may significantly influence the overall cost of assay optimization and subsequently the cost *per* sample analyzed. Another aspect is that nonlabeled primers are required in template preparation steps of many enzymatic assays. In these cases, conventional CE with LIF detection cannot be utilized for controlling quality of intermediate unlabeled PCR products. As a result, several approaches were developed to overcome the need of labeled PCR primers, including post-PCR labeling [3] and, in particular, labeling by intercalation [4].

Using intercalating dyes for DNA detection and quantification has been originally applied in fluorometry [5–7], fluorescence microscopy [8], and later refined for visualization of DNA bands separated by slab-gel electrophoresis [9]. Due to its universality, the technique quickly found its use for detection in other areas, such as flow cytometry, [10] melting-curve analysis [11], and more recently real-time PCR [12]. The principle of detection by intercalation is based on a substantial increase in fluorescence signal emitted from the dye molecules upon their noncovalent complexation with individual nucleotides composing the DNA chain. A typical number of dye molecules interacting with each nucleotide expressed as dye to basepair ratio can be ranging from 0.001 to 0.5, depending on the stoichiometry of the interaction. The amount of generated fluorescence signal is naturally

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higher for longer fragments that are capable of incorporating a higher number of the intercalator molecules compared to the short fragments [13]. A higher concentration of the dye naturally leads to a better signal due to more abundant complexation with the DNA, but it also increases the background noise as a result of the intercalator's native residual fluorescence. It is therefore important to optimize the dye concentration for achieving the best sensitivity in terms of the S/N [14]. Although the original monomeric intercalating dyes (*e.g.*, ethidium bromide) are exhibiting fluorescence predominantly upon interaction with dsDNA, the subsequently developed dimeric dyes (*e.g.*, SYBR Green or PicoGreen) are capable of producing fluorescent signal upon interaction with both, double- as well as single-stranded nucleic acid chains [15, 16]. This recently opened a range of potential applications to other methods such as RNA analysis and gene expression by microarrays [17].

Intercalation has been used as a tool for detection since the early days of CE separation of DNA fragments [18]. It was shown that, similar to slab gel electrophoresis, the intercalator–DNA interaction induces shifts in migration times by changing weight and charge of the DNA fragment [19, 20]. It was also demonstrated that the presence of intercalator might improve consistence of migration (size dependence) by suppressing secondary-structure effects [21]. Modern dimeric dyes have also been utilized for detection of RNA [22]. Intercalation has been widely utilized on home-made CE systems [23] and, recently, also on a commercial single-capillary instrument [24, 25]. In a typical CE experiment, the dye is added to the sieving polymer matrix before filling the capillaries [23–25]. This method is straightforward when using home-made polymers; however, mixing the dye into viscous commercially available gels is problematic, especially, considering that a fresh batch of intercalating dye usually needs to be prepared prior to each analysis. The inconvenience of mixing the intercalator into the sieving gel matrix can be avoided by preincubating the DNA sample with the dye prior to analysis [26]. During the run, however, the DNA–dye complex tends to break up, which may result in a loss of signal. To improve the DNA–dye complex stability, a tetrapentylammonium has been suggested as a stabilizing agent additive into the running buffer [27].

The main purpose of the presented work was to investigate an alternative configuration for separation of DNA fragments with detection by intercalation. The suggested approach is based on dynamic interaction of unlabeled DNA fragments with intercalator molecules present within the separation matrix rather than relying on stability of premixed DNA–dye complex. However, instead of mixing the intercalator directly into the sieving gel matrix we propose a scheme in which the dye is only added to the anode electrolyte container. At optimum CE conditions, the positively charged intercalator ions move into the capillary and interact with the negatively charged DNA fragments in a counter-current migration fashion, resulting in intercalating effect at the detector.

Utilization of dynamic interactions with opposite-migrating agents during electrophoretic separation is not a brand new concept in CE. The method is routinely applied to induce a secondary separation principle to compounds that would otherwise comigrate. The most profound example is in chiral CE separations, where compounds may be separated upon interaction with charged buffer additive (chiral selector, *e.g.*, CD) migrating against the sample zones [28, 29]. A CE system based on this principle found its use in combination with detection by MS, where the counter-migrating chiral selector induces separation in the capillary, but does not interfere with detection by entering the MS chamber [30]. Similarly, interaction between a receptor and ligand can be utilized in affinity CE, with either of the agents serving as a counter-migrating buffer additive for screening of peptide and other libraries for potential drug targets [31]. Counter-current migration was also utilized for monitoring of DNA–ligand binding [32] or testing of properties of polymeric sieving matrices [33].

In the present work we demonstrate high sensitivity and linearity of the counter-current intercalation approach, offering a major advantage by utilizing dye-free separation gel matrix. We attempt to demonstrate potential for usability in routine applications performed on a commercial capillary-array sequencer.

2 Materials and methods

2.1 Chemicals

All CE experiments were performed using standard buffers and components supplied by the CE instrument manufacturer (Amersham Biosciences, Piscataway, NJ, USA). pUC18/*Hae*III digest was obtained from Sigma-Aldrich (St. Louis, MO, USA). Ethidium bromide was obtained from Top-Bio (Prague, Czech Republic), SYBR Green I and SYBR Green II dyes were from Molecular Probes (Eugene, OR, USA).

2.2 CE

All CE experiments were performed on MegaBACE™ 1000 96-capillary DNA analysis system (Amersham Biosciences). The instrument was equipped with an automated loading robot Caddy 1000 (Watrex Praha, Prague,

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Czech Republic), to allow unattended insertion of sample plates during overnight operation. CE running conditions are noted in the text and figure captions. Ethidium bromide was added to the anode buffer at a concentration of 1 ng/μL. An ideal dilution for SYBR Green I and II dyes was 1:50 000 (*i.e.*, five times below the manufacturer recommended dilution). Chemical structures were created using Structure Editor version 1.0, a drawing utility provided by HighChem (Bratislava, Slovakia).

3 Results and discussion

A prerequisite for counter-current migration principle is ability of the complexing agent to migrate opposite to the direction of the separation. In case of intercalation, this condition is met, since most dyes bear a positive charge. An example is illustrated in Fig. 1 with chemical formulas of two common intercalating dyes, ethidium bromide and SYBR Green I [34]. Both dyes exhibit positive charge at pH 8–9 used in buffers for DNA CE. A newer generation intercalating dyes also often exhibit positive charge. One of most popular intercalators, SYBR Green II, is apparently a dimeric dye; however, its chemical structure has not yet been publicly revealed. The most important feature of SYBR Green II is its higher fluorescence yield in comparison to, for example, ethidium bromide and SYBR Green I. In addition, SYBR Green II is able to form fluorescent complexes with double- as well as single-stranded nucleic acid chains and is, therefore, widely used for detection of DNA as well as RNA. Since we have confirmed a positive charge at SYBR Green II, all experiments in this paper were performed principally with SYBR Green II. In addition, we have performed parallel experiments with ethidium bromide as the "golden" intercalation standard.

The schematic illustration of the principle of intercalation by counter-current migration is presented in Fig. 2. The dye is only added to the buffer in anode reservoir and by electrophoresis the positively charged molecules migrate into the capillary and against the separating unlabeled DNA fragments. Depending on the mobility of the intercalator and the detector position from the capillary end, it is necessary to perform a short pre-electrophoresis prior to sample injection to allow the dye to reach detector before the first DNA fragments. A presence of the intercalator in the detector can be noticed by a slight increase in baseline signal due to the residual native fluorescence of the unbound intercalator. The moving boundary of the dye can be noticed as an increase of background signal during the pre-electrophoresis as shown in Fig. 3. The signal was monitored during the pre-electrophoresis step on a system with 50 cm capillary array having a detector positioned 10 cm from the end (*i.e.*, 40 cm separation distance). At a voltage of 10 000 V the migration time of ethidium bromide was 12 min, the migration time of SYBR Green II was 19 min. This corresponds to an approximate electrophoretic mobility of 1.44×10^{-8} m^2/V × s for ethidium bromide and 0.88×10^{-8} m^2/V × s for SYBR Green II, both less than a half of the mobility of short DNA fragments. As a result of these measurements a pre-electrophoresis time of 10 min is sufficient, considering that the DNA fragments appear at the detector after 10 min following the injection. A pre-electrophoresis step is already performed on commercial capillary genetic analyzers to clear the polymer matrix from small molecular impurities [35]. The "matrix conditioning" can thus be performed with the intercalator-enriched buffer in the anode reservoir to maintain the overall turnaround time.

A typical result of multicapillary CE separation of unlabeled DNA fragments is presented in Fig. 4. An equimolar mixture of pUC18/*Hae*III restriction fragments (unlabeled)

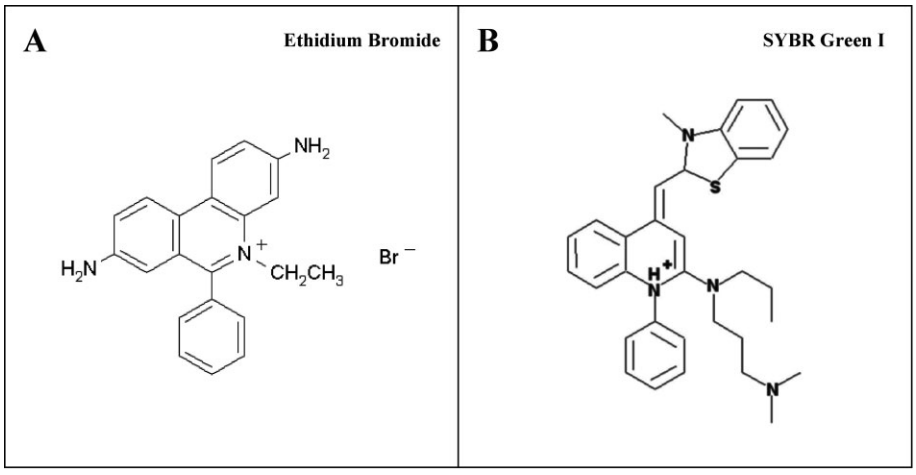

Figure 1. Structural formulas of intercalation dyes commonly used in CE; ethidium-bromide (A) and SYBR Green I (B) (obtained from [34]).

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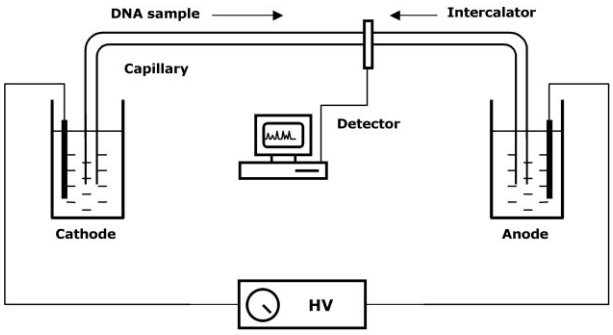

Figure 2. Scheme of a CE experiment with fluorescent detection by counter-current migration of an intercalating dye.

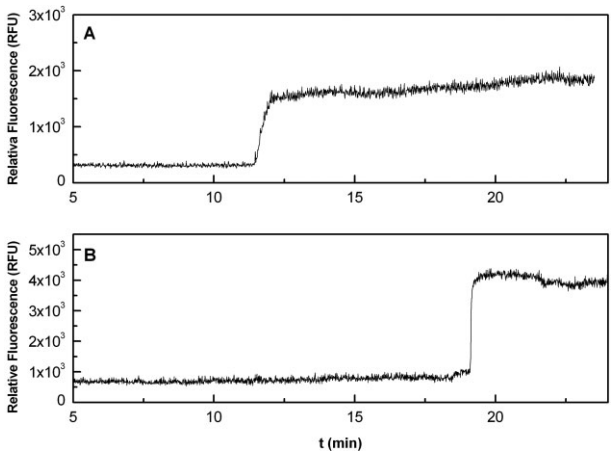

Figure 3. Determination of the pre-electrophoresis time needed for the intercalating dye to reach the detector. An increase in signal is recorded due to native background fluorescence of the free intercalating dye entering the detector from the anodic end with ethidium bromide (A) and SYBR Green II (B).

was injected into a capillary array following a 10-min pre-electrophoresis with either ethidium bromide (A) or SYBR Green II (B) additive in the anodic reservoir. In both cases all of the separated fragments could clearly be identified in all capillaries (8 representative capillaries out of 48 used are shown). An increase in peak intensity with the length of the DNA fragment is noticeable for both dyes. This is expected since more intercalator molecules are likely to be bound to a large DNA fragment compared to a short fragment. In addition, the intercalator concentration in the anodic reservoir needs to be selected properly to produce maximum signal without increasing noise levels stemming from native background fluorescence of the free unbound dye.

The ability to quantify individual DNA fragments is fundamentally important in fragment analysis by CE. For the presented method of counter-current intercalation, we

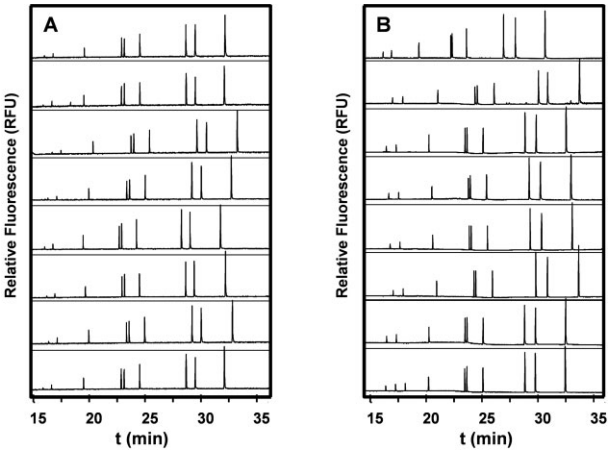

Figure 4. Comparison of separation of pUC18/*Hae*III digest performed by multicapillary electrophoresis with detection by counter-current migration of intercalating dye using ethidium bromide (A) and SYBR Green II (B). CE conditions: Tris-TAPS buffer (pH 8.2), Injection: 100 sec at 1 kV, Separation: 60 minutes at 10 kV. Peak sizes: 80 bp, 102 bp, 174 bp, 257 bp, 267 bp, 298 bp, 434 bp, 458 bp and 587 bp. (8 out of 48 capillaries shown).

have tested the signal response to various concentrations of a set of DNA fragment sizes using the pUC18/*Hae*III restriction digest as a model fragment mixture. A series of runs was performed with pUC18/*Hae*III concentrations ranging from 0.1 to 50 pg/μL. In order to eliminate peak intensity distortion from injection stacking effects, the different concentrations were created by dissolving the pUC18/*Hae*III standard in Tris-EDTA buffer (1 mM Tris, 0.1 mM EDTA). The obtained data is presented in Fig. 5, showing plots for peak areas at various pUC18/*Hae*III standard concentrations. The upper plot (Fig. 5A) was obtained with ethidium bromide, the lower (Fig. 5B) plot with SYBR Green II, each added only to the anode buffer. As expected, in both cases the signal intensity increases with the fragment length. At low concentrations, the level of signal (peak areas) is directly proportional to the fragment bp-length. At high concentrations, the signal is no longer proportional to the fragment size, which could be contributed to either a local depletion of the intercalator or a simple injection overload. Such saturation effect must be avoided when quantifying DNA. For the selection of 100–700 bp fragments the linear concentration of the pUC18/*Hae*III mixture was 0.05–0.50 ng/μL for ethidium bromide and 0.01–0.06 ng/μL for SYBR Green II. Indeed, due to the electrokinetic injection method these parameters depend on various factors such as pH, sample ionic strength, or temperature, which are usually specific to the sample used. At the given injection conditions, the experimentally observed LOD for a 102 bp fragment was

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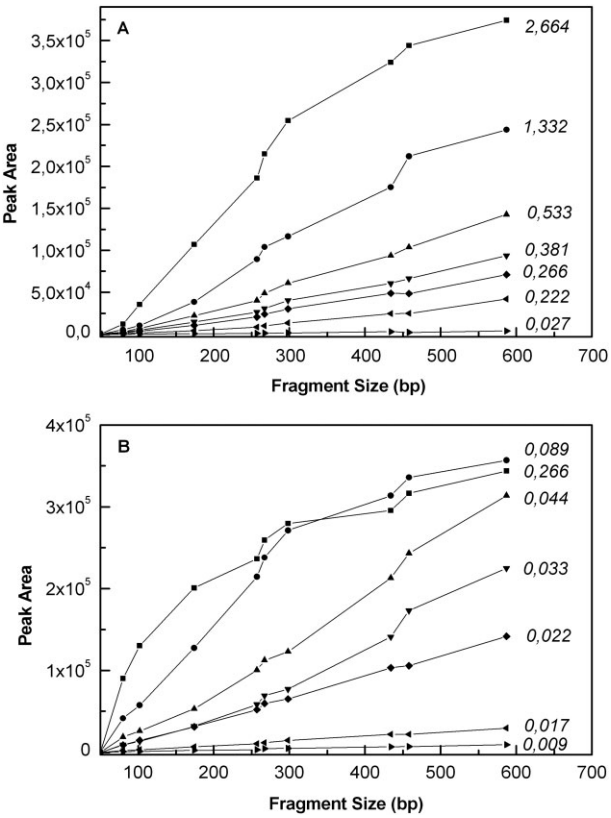

Figure 5. Peak signal intensity as a function of the DNA size at different concentrations of the pUC18/*Hae*III standard fragment mixture using ethidium bromide (A) and SYBR Green (B) intercalators for labeling by counter-current migration. Sample concentrations are shown in ng/μL. CE conditions: Tris-TAPS buffer (pH 8.2); injection: 100 s at 1 kV; separation: 60 min at 10 kV.

25 and 4 pg/μL for a 587 bp fragment with ethidium bromide. At the same injection conditions, with SYBR Green II the overall sensitivity was better, resulting in an LOD of 4 pg/μL for the 102 bp fragment and 0.4 pg/μL for the 587 bp fragment. All concentrations relate to the pUC18/*Hae*III standard. Run-to-run reproducibility of the detected peak areas of the individual fragments in the mixture was evaluated using a series of five runs. The resulting RSD values were always in the range from 12 to 20%, showing a good performance of the system. An overview of all experimental parameters of the two tested dyes suitable for CE with intercalation by counter-current migration is shown in Table 1.

4 Concluding remarks

In the present work, we have demonstrated a simple technique for detecting unlabeled DNA fragments on a multicapillary-array sequencer. The DNA fragments are detected upon their intercalation with a positively charged dye entering the capillary from the detector end and migrating in direction opposite to the sample migration. A short pre-electrophoresis prior to the sample injection is necessary to allow the intercalating cations reach the detector before the counter-migrating sample anions. This can be done simply as a part of a standard protocol during the "conditioning" step, performed on all commercial DNA sequencers. The duration of such pre-electrophoresis may vary based on the distance between anode buffer reservoir and the detector (depending on the instrument used). It is preferable to maintain a fresh solution of intercalator-containing anodic buffer to avoid dye depletion. With most commercial-array sequencers this does not pose a problem, since the anode buffer vials are either replaced prior to each run or a large buffer container (50–80 mL) is used at the anode. The narrow linearity range (one order of magnitude) could be contributed to the limited dynamic range of the capillary-array sequencer used in this study, rather than to the used intercalating dyes. A comparative study on different instruments is now being prepared. At given electrokinetic injection conditions, the LODs within the linearity range (*i.e.*, without utilizing sample stacking) were on pg/μL levels, which typically represent 10 000–100 000 dilutions of unpurified PCR product.

The main advantage of the presented approach is elimination of a need to premix the dye into the gel matrix. This represents a significant advantage, since regular sieving matrices can be directly applied for analysis on unlabeled fragments. Addition of the intercalator to the anodic buffer can be easily done just prior to analysis, eliminating potential problems with premixed dye matrix undergoing photobleaching. The main application is in high-throughput analysis of PCR fragments requiring large numbers of fluorescently labeled primers, such as in mutation detec-

Table 1. Overview of basic parameters of two intercalating dyes used for labeling by counter-current migration

	Ethidium bromide	SYBR Green II
λ excitation, nm	532 (green)	488 (blue)
λ detection, nm	610	520
Electrophoretic mobility, $m^2/V \times s$	1.44×10^{-8}	0.88×10^{-8}
Elution time at 10 kV (40 cm detection distance), min	12	19
Linearity range, ng/μL	0.05–0.50	0.01–0.06
Noise level,%	0.25	0.33
LOD for 587 bp, ng/μL	0.004	0.0004
LOD for 102 bp, ng/μL	0.025	0.004

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tion or SNP screening. The technique is directly applicable on most currently used capillary-array systems and can potentially be applied also in microfabricated CE devices (chip CE).

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Analysis of genetic events in 17p13 and 9p21 regions supports predominant monoclonal origin of multifocal and recurrent bladder cancer

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Summary

Clonality was tested in 86 tumours from 25 patients with recurrent and multifocal superficial bladder transitional cell carcinomas (TCCs) using the analysis of TP53 mutations and of LOH in the 17p13 and 9p21 regions. Tumours from the majority of individuals showed either absence or presence of the same TP53 mutation and/or an identical LOH pattern, with the same allele lost in all tumours. Only two pairs of tumours from two patients had discordant findings, which were incompatible with monoclonality. Therefore, our results rather support the monoclonal model of development of highly recurrent superficial bladder TCCs.
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Keywords: Transitional cell carcinoma (TCC); Bladder cancer; Clonality; Recurrent cancer; LOH; TP53 mutations

1. Introduction

Bladder cancer is the second most common malignancy of the genitourinary tract with a male to female ratio of 3:1 [1]. About 90% of all bladder cancers are transitional cell carcinomas (TCCs) derived from the urothelium, and more than 80% of them are superficial, non-muscle-invasive tumours (classified as Ta, T1 or Tis). Approximately 70% of all patients with superficial bladder TCCs develop recurrences after transurethral resection (TUR), and in 10–20% of patients the progression to muscle invasion (T2–T4) occurs [2]. Nearly 30% of all patients present at diagnosis with multifocal disease—simultaneous occurrence of several spatially distinct tumours at different sites of the bladder wall [3].

Simultaneous or metachronous development of multiple superficial bladder TCCs evokes the question of possible monoclonal nature of these tumours. During the last years, two hypotheses have been proposed: the monoclonal hypothesis, and the field cancerization hypothesis. According to the monoclonal model, the progeny of a single malignant cell proliferates and spreads throughout the urothelium either via intraluminal seeding (when the transformed cell is fully released from the primary tumour), or via intraepithelial migration of the malignant cell. On the contrary, the field cancerization model proposes independent

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transformation of numerous urothelial cells at multiple sites as a result of accumulation of carcinogenic events, leading to the growth of multiple unrelated tumours [4].

Various molecular genetic methods or their combinations can be used to determine the clonality of multiple synchronous or recurrent tumours in one patient including X-chromosome inactivation analysis, molecular cytogenetic techniques (FISH, CGH), loss of heterozygosity (LOH) analysis, and single-gene mutation analysis [5–11]. Several studies published up to now presented evidence both for and against the monoclonal model of bladder cancer, but the majority of molecular studies favoured the monoclonal origin of multiple tumours in one patient. Most of these studies concentrated on advanced-stage invasive carcinomas [5–8]. On the other hand, several studies, which found evidence for the existence of more than one tumour clone, particularly in the early stages of bladder carcinoma, supported the field cancerization hypothesis [9–11].

We attempted to address this question by the analysis of genetic events in the 17p13 and 9p21 regions of the human genome, harbouring the TP53 and CDKN2A genes known to play a role in bladder tumorigenesis [12], in a large series of well characterized multifocal and recurrent superficial bladder TCCs. TP53 gene mutations and LOH at several TP53 and CDKN2A intragenic and extragenic polymorphic DNA markers were analysed in a total of 86 tumours from 25 patients sampled during a period of 5 years. The patients were selected for highly recurrent disease, and the study was particularly focused on the analysis of the genetic profile of the early stages of the disease. Our results support the monoclonal model of development of multifocal and recurrent superficial bladder TCCs.

2. Materials and methods

2.1. Patients and samples

The study comprised 25 patients with Ta or T1 bladder TCCs (22 males and three females, mean age 69.8 years) treated with TUR in the period from April 2000 to May 2002 in whom one or more tumour recurrences during the follow-up period (till December 2004) appeared (Table 1). DNA testing was performed after obtaining of informed consent from each patient. TUR was performed using standard techniques with the removal of all visually identifiable tumour tissue. After TUR, the patients were followed and treated according to the current convention and depending on the general health status and age of each individual patient (in G3 and multiple T1G2 tumours intravesical bacillus Calmette-Guerin immunotherapy and in TaG2 or multiple G1 tumours intravesical chemotherapy were administered). Tumour progression was defined as the development of muscle invasive disease, and disease generalization as the appearance of distant metastases.

In total, tissue samples from 86 different tumours were obtained. Blood samples were available from all patients for the analysis of marker informativity. Tumours were classified according to the TNM system [13]: 50 superficial urothelial tumours were classified as Ta, 32 tumours as T1, and four recurrent tumours were muscle invasive (three T2 and one T3). Tumour grading was performed according to the criteria defined by the WHO International Classification of Tumours [14]: 21 tumours were G1, 58 tumours were G2, and seven were G3 (Table 1). The tumour samples were frozen in liquid nitrogen and stored at −70 °C until DNA extraction.

2.2. Analysis of TP53 mutations and of LOH in the 17p13 and 9p21 regions

DNA isolation from the peripheral blood lymphocytes and from the frozen tissue samples was performed using the QIAamp DNA Blood Midi Kit and QIAamp DNA Mini Kit (Qiagene). TP53 mutations were screened in exons 5–9 using direct sequencing of PCR products as described previously [15]. LOH analysis was performed for at least one of seven polymorphic DNA marker loci in the TP53 gene region of 17p13, and for at least one of three markers in the CDKN2A gene region of 9p21 (Table 2). Genotypes for TP53 codons 72 and 213 were analysed by restriction digestion [16,17]. The other markers were microsatellites (STRs), and their typing was performed using capillary electrophoresis with laser-induced fluorescence detection. FAM-labelled primers were used for typing of the 9p21 markers. Amplification of the 17p13 markers was performed with non-labelled primers, and the resulting unlabelled PCR products were detected on a multicapillary sequencer using counter-current migration approach in which intercalation takes place upon contact of the DNA fragments and opposite-migrating SYBR Green II dye molecules [18]. The STR polymorphism in polyA tail of one of the Alu sequences in intron 1 of the TP53 gene [19] was amplified by a semi-nested PCR. LOH was determined by the comparison of the genotypes from blood lymphocyte and tumour DNAs in individual patients.

2.3. Statistical analysis

The correlation between the presence of genetic alterations on 17p13 or 9p21 and disease progression or disease generalization was analysed by the Fisher's factorial test.

Table 1
Results of the mutation and LOH analyses

Patient/tumour[a]	Date[b]	Diag.[c]	17p13		9p21
			TP53[d]	LOH[e]	LOH
01(54/M)				TP53i1	D9S1748
1	8/00	TamG2	Wt	LOH-A2	RH
2	1/01	TamG2	Wt	LOH-A2	RH
3	5/01	TamG2	Wt	LOH-A2	RH
4	6/01	T1G2	Wt	LOH-A2	RH
disease progression and generalization 2/02, death 11/03					
02(62/M)				TP53c213	D9S1748
1.1	9/00	T1mG2	Hom	LOH-A2	LOH-A2
1.2	9/00	T1mG2	Hom	LOH-A2	LOH-A2
2.1	2/01	TamG2	Hom	LOH-A2	LOH-A2
2.2	2/01	TamG2	Hom	LOH-A2	LOH-A2
3.1	7/01	T3mG3	Hom	LOH-A2	LOH-A2
3.2	7/01	T2mG2	Hom	LOH-A2	LOH-A2
4	9/01	T1mG2	Hom	LOH-A2	LOH-A2
5	5/02	T1mG2	Hom	LOH-A2	LOH-A2
disease progression 7/01, generalization 5/02, death 4/03					
03(76/M)				D17S578	D9S1748
1	5/01	T1mG2	Wt	RH	RH
2	9/01	TamG1	Wt	RH	RH
3	3/02	T1mG1	Wt	RH	LOH-A1
4	9/02	TamG2	Wt	RH	RH
5	1/03	TamG2	Wt	RH	LOH-A1
disease progression 6/04, alive					
04(72/M)				TP53c72	D9S1748
1	9/00	TamG1	Wt	RH	RH
2	10/01	T1mG1	Wt	RH	RH
3	5/02	TamG1	Wt	RH	RH
4	3/03	TamG1	Wt	RH	RH
5	5/04	TamG1	Wt	RH	RH
no disease progression, alive					
05(80/F)				D17S578	D9S1748
1	5/00	T1mG1	Wt	RH	RH
2	6/01	TaG3	Wt	RH	RH
3	9/01	TaG2	Wt	RH	RH
4	3/02	TamG2	Wt	RH	RH
disease remission since 3/02, alive					
06(67/M)				TP53c72	D9S157
1	9/00	T1G2	Wt	RH	RH
2	9/02	TamG2	Wt	RH	LOH-A2
3	3/03	TamG2	Wt	RH	LOH-A2
disease generalization 5/03, death 11/03					
07(63/M)				TP53i1	D9S1748
1	9/00	T1mG2	Wt	RH	RH
2	4/01	TamG2	Wt	RH	RH
disease remission since 4/01, alive					
08(78/M)				TP53c72	D9S171
1.1	7/00	TamG2	Hom	LOH-A1	LOH-A1
1.2	7/00	TamG2	Hom	LOH-A1	LOH-A1
2.1	5/01	T1mG2	Hom	LOH-A1	LOH-A1
2.2	5/01	T1mG2	Hom	LOH-A1	LOH-A1
3	10/01	T1G3	Hom	LOH-A1	LOH-A1
4	6/02	T1G2	Hom	LOH-A1	LOH-A1

(*continued on next page*)

Table 1 (*continued*)

Patient/tumour[a]	Date[b]	Diag.[c]	17p13		9p21
			TP53[d]	LOH[e]	LOH
disease progression and generalization 5/03, death 10/03					
09(75/M)				D17S960	D9S171
1	2/01	TamG2	Wt	RH	LOH-A1
2	4/01	T1G2	Wt	RH	LOH-A1
disease remission since 4/01, alive					
10(75/M)				TP53c72	D9S1748
1	10/01	T1mG2	Wt	RH	RH
2	3/02	T1mG2	Wt	RH	RH
3	9/04	T1Mg2	Wt	RH	RH
no disease progression, death 10/04 (stroke)					
11(75/F)				D17S578	D9S1748
1.1	11/01	TamG1	Wt	RH	RH
1.2	11/01	TamG1	Wt	RH	RH
2.1	9/02	TamG1	Wt	RH	RH
2.2	9/02	TamG1	Wt	RH	RH
disease remission since 9/02, death 9/03 (other causes)					
12(59/M)				TP53mut	D9S1748
1	7/01	T1mG2	Het	RH	LOH-A1
2	9/01	T1G2	Het	RH	LOH-A1
further tumour recurrences, no disease progression, alive					
13(68/M)				D17S578	D9S1748
1	5/02	T1G2	Wt	RH	RH
2	2/03	T1G2	Wt	RH	RH
further turmour recurrences, no disease progression data					
14(58/M)				TP53i1	D9S1748
1	3/02	T1G2	hom	LOH-A1	RH
2	10/02	T1G2	hom	LOH-A1	RH
disease progression 6/03, remission after cystectomy, alive					
15(77/M)				TP53c72[f]	D9S171
1	3/02	T1mG2	wt	RH	LOH-A1
2	5/04	TaG1	wt	LOH-A2	RH
no disease progression, alive					
16(69/M)				D17S906	D9S1748
1.1	5/00	T1mG3	wt	RH	RH
1.2	5/00	T1mG3	wt	RH	RH
2.1	9/00	T1mG3	wt	RH	RH
2.2	9/00	T1mG3	wt	RH	RH
disease progression and generalization 11/00, death 6/01					
17(82/M)				TP53c72	D9S171
1	4/00	T1mG2	Wt	RH	LOH-A2
2	1/01	TamG1	Wt	RH	LOH-A2
no information since 1/01					
18(74/M)				TP53c72	D9S1748
1.1	6/00	TamG2	Wt	RH	RH
1.2	6/00	TamG2	Wt	RH	RH
2.1	9/02	TamG2	Wt	RH	LOH-A1
2.2	9/02	TamG2	Wt	RH	LOH-A1
3	¼	T2mG2	Wt	RH	RH
4	5/04	T2mG2	Wt	RH	RH
5	12/04	T1mG2	Wt	RH	RH
disease progression 01/04, generalization 12/04, alive					
19(78/M)				D17D1353	D9S1748
1	8/00	TaG2	Wt	RH	RH

Table 1 (*continued*)

Patient/tumour[a]	Date[b]	Diag.[c]	17p13		9p21
			TP53[d]	LOH[e]	LOH
2	4/01	T1G2	Wt	RH	RH
further tumour recurrences, no disease progression, alive					
20(67/M)				D17S960	D9S1748
1	1/01	TamG2	Wt	RH	LOH-A1
2	9/02	TamG1	Wt	RH	LOH-A1
disease remission since 9/02, alive					
21(81/F)				TP53i1	D9S1748
1.1	7/01	TamG2	Wt	LOH-A2	RH
1.2	7/01	TamG2	Wt	LOH-A2	RH
2.1	11/02	TamG2	Wt	LOH-A2	LOH-A1
2.2	11/02	TamG2	Wt	LOH-A2	LOH-A1
disease progression 2/03, remission after TUR					
22(66/M)				TP53c72	D9S1748
1	12/00	TamG2	Wt	LOH-A2	LOH-A2
2	7/01	TaG2	Wt	LOH-A2	LOH-A2
disease remission since 7/01, alive					
23(79/M)				TP53c72[g]	D9S1748
1.1	3/01	TamG2	Wt	RH	RH
1.2	3/01	TamG2	Wt	RH	RH
2	3/03	TamG1	Wt	LOH-A2	RH
3	9/04	TamG1	Wt	RH	RH
4	12/04	TamG2	Wt	LOH-A2	RH
no disease progression, alive					
24(44/M)				D17S578	D9S1748
1	9/01	TaG1	Wt	RH	RH
2	3/03	TamG2	Wt	RH	RH
disease remission since 3/03, alive					
25(65/M)				TP53i1	D9S1748
1	8/01	TamG1	Wt	RH	LOH-A1
2	3/03	TamG1	Wt	RH	RH
further tumour recurrences, no disease progression, alive					

[a] Patient number (age/sex)/tumour number. Tumour numbers with decadic points indicate multiple synchronous tumours.

3. Results

3.1. Clinical course of the disease

During the course of the study, five patients out of the total of 25 (20%) showed tumour progression and disease generalization, three patients (12%) showed tumour progression but no disease generalization, and one patient (4%) underwent disease generalization without any detectable muscle invasive disease in the bladder (histologically confirmed lung metastases in case 06). Out of these nine patients, five died from bladder cancer, two are alive with disease progression, and two are currently in disease remission after cystectomy and TUR, respectively. Of the remaining 16 patients, eight had further recurrences (one of them (case 10) died from other causes), three patients remained in disease remission since 2001, three since 2002 (but one (case 11) died from other causes), and one patient since 2003. No information is available about one patient (case 17) since 2001. Complete clinical data for each patient is summarized in Table 1.

3.2. TP53 mutations

TP53 mutations were identified in tumours from four of the total of 25 patients (16%). An identical mutation was always identified in all tumours of each particular patient (Table 1). In three patients the mutations were in a homo- or hemizygous state, and no wild type sequence could be detected at the mutation site. The fourth mutation was in a heterozygous state, and the tumours carried both the wild type and the mutated sequence. Two missense homo/hemizygous mutations were observed: an AAG to AAC (Lys132Asn) mutation in exon 5 in eight tumours of patient 02, and an ATG to ATC (Met246Ile) mutation in exon 7 in two tumours of patient 14. A heterozygous missense mutation TAT to TGT (Tyr220Cys) in exon 6 was found in two tumours of patient 12. Finally, a homo/hemizygous deletion of three C nucleotides in codons

Table 2
Polymorphic DNA markers used for LOH analysis

Name	Localization	Type	PCR primers	Method
17p13 region				
TP53c72	TP53 exon 4, codon 72	CGC>CCC (Arg>Pro)	CCTCTGACTGCTCTTTTCACCC GAAGTCATGGAAGCCAGCCC	Acc II digestion (or sequencing)
TP53c213	TP53 exon 6, codon 213	CGA>CGG (silent)	TGCCCTGACTTTCAACTCTGTC CCACTGACAACCACCCTTAACC	Taq I digestion (or sequencing)
TP53i1	TP53 intron 1	$(AAAAT)_n$	CCAGCACTTTCCTCAACTCTAC AACAGCTCCTTTAATGGCAGGC AGATAGTGCCACTGTACTCCAG	Semi-nested PCR +Fragmentation analysis (unlabelled product)
D17D1353	26 kb proximal to TP53	$(CA)_n$	CTGAGGCACGAGAATTGCAC TACTATTCAGCCCGAGGTGC	Fragmentation analysis (unlabelled product)
D17S960	315 kb distal to TP53	$(CA)_n$	TGATGCATATACATGCGTGCAC TAGCGACTCTTCTGGCACAG	Fragmentation analysis (unlabelled product)
D17S578	750 kb distal to TP53	$(CA)_n$	CTATCAATAAGCATTGGCCT CTGGAGTTGAGACTAGCCT	Fragmentation analysis (unlabelled product)
D17S906	875 kb distal to TP53	$(AAAG)_n$	GAGCAAGATTCTGTCAAAAGAG TCTAGCAGAGTGAAACTGTCTC	Fragmentation analysis (unlabelled product)
9p21 region	CDKN2A intron 1	$(CA)_n$	CACCTCAGAAGTCAGTGAGT	Fragmentation analysis
D9S1748			GTGCTTGAAATACACCTTTCC	(fluorescently labelled product)
D9S171	2.5 Mb prox. to CDKN2A	$(CA)_n$	AGCTAAGTGAACCTCATCTCTGTCT ACCCTAGCACTGATGGTATAGTCT	Fragmentation analysis (fluorescently labelled product)
D9S157	4.5 Mb distal to CDKN2A	$(CA)_n$	AGCAAGGCAAGCCACATTTC TGGGGATGCCCAGATAACTATATC	Fragmentation analysis (fluorescently labelled product)

127–128 in exon 5 resulting into a deletion of proline in position 128 was identified in six tumours of patient 08.

3.3. LOH analysis

Table 1 shows the summary of the LOH analysis. Genotyping of peripheral blood lymphocyte DNA showed that each of the patients was informative for at least one marker from the 17p13 region and at least one marker from 9p21. Codon 72 of the TP53 gene, the constitutional genotype of which may be associated with some cancers including bladder cancer [20], was heterozygous (Arg/Pro) in nine patients (36%), homozygous (Pro/Pro) in one patient (case 20, 4%), and homozygous (Arg/Arg) in the remaining 15 patients (60%). Constitutional heterozygosity of codon 213 in TP53 exon 6 was found in one patient (case 02, 4%).

According to the LOH data, the patients could be classified into two groups: group A with complete concordance of genetic alterations in all their multifocal and recurrent tumours in all genetic markers tested, and group B with discordant results (Table 1). Of the total of 25 patients, 18 (72%) belonged to group A, and seven (28%) to group B. In group A, retention of heterozygosity (RH) in all tumours on both 17p13 and 9p21 was found in nine patients (cases 04, 05, 07, 10, 11, 13, 16, 19, 24). RH on 17p13 and identical LOH pattern on 9p21 were detected in all tumours of four patients (cases 09, 12, 17, 20). Identical LOH pattern on 17p13 and RH on 9p21 were found in tumours of two patients (cases 01, 14). Finally, identical LOH pattern on both 17p13 and 9p21 was detected in all tumours of three patients (cases 02, 08, 22) (Table 1).

In group B, the most frequent observation was concordant RH on chromosome 17p13 in all tumours and discordant findings on 9p21 (four patients, cases 03, 06, 18, 25). One patient had concordant LOH on 17p13 and discordant findings on 9p21 (case 21), one patient had concordant RH on 9p21 and discordant findings on 17p13 (case 23), and one patient (case 15) had discordant findings at both loci (Table 1).

In total, LOH in one or more markers was identified in 45 of 86 tumours (52.3%) from 16 of 25 patients (64%). Nine of these 16 patients (56.3, 36% from the total of 25) demonstrated an identical LOH pattern in all their tumours. The LOH status on 17p13 was always in agreement with the TP53 mutation status. Eight patients out of the total of nine with disease progression and/or generalization had LOH in at least one marker: five had an identical LOH pattern in all tumours, and three underwent a conversion from heterozygosity to LOH.

3.4. Correlation with clinical data

No statistically significant correlation between TP53 mutations or LOH on 17p13 or on 9p21 and disease progression or disease generalization was found.

4. Discussion

The mutation analysis of the TP53 gene and LOH analysis at multiple marker loci in the 17p13 and 9p21 regions can detect specific genetic alterations in tumours, and identical TP53 mutation and/or LOH patterns in tumours from one patient could be a strong indicator of monoclonality.

TP53 mutations were confirmed in four of 25 patients (16%) with superficial bladder TCCs, and all tumours from each of these patients always carried the same mutation. One of the mutations was a deletion of three C nucleotides in a stretch of four consecutive C's in TP53 codons 127–128, possibly due to replication slippage. The remaining mutations included two G to C transversions, and one A to G transition. No common spontaneous C to T transitions in CpG dinucleotides were involved. With the exception of the deletion mutation, all mutations found in our patients have already been described in tumours [21]. High frequency of G to C transversions has been described in bladder cancer, and may be associated with environmental influences (e.g. smoking) [22]. TP53 mutations are more frequent in high-stage and high-grade tumours than in low-grade superficial TCCs, and this is reflected in a clear association with outcome [23]. Even though no significant correlation between the detection of molecular alteration and the tumour recurrence in patients with superficial bladder TCCs could be found [24], it has already been suggested that TP53 could be useful for the selection of patients with high-risk superficial disease in whom aggressive therapy is appropriate [25]. This tendency is also supported by our data. Although statistical significance was not reached, three patients with homozygous TP53 mutations had disease progression, and two of them died from bladder cancer.

All three possible genotypes of the TP53 codon 72 polymorphism were observed in lymphocyte DNAs of our patients, and their frequencies are within the range described for Caucasian populations [26]. Chen et al. [20] examined the distribution of this polymorphism in bladder cancer and found no differences between control subjects and patients, but proline homozygotes were more frequently found in the carriers of invasive tumours compared to patients with non-invasive tumours (25 and 2.9%, respectively). The clinical course of the disease in the only proline allele homozygote from our series (case 20) does not follow this trend. LOH in codon 72 was found in tumours of three patients from our series, and two of them lost the arginine allele.

In total, we were able to detect genetic alterations in the 17p13 and/or 9p21 regions in more than a half of tumours and in almost 2/3 of patients. The LOH analysis cannot detect point defects or possible methylation silencing of the CDKN2A gene, and genetic alterations in low-frequency tumour clones on the background of other DNA. Direct sequencing of PCR products as a method for detection of TP53 mutations in tumours may suffer from limited sensitivity, as up to 30–70% of mutated DNA must be present in the sample to allow reliable detection of a mutation [27]. The identification of three homo- or hemizygous TP53 mutations without any background of normal sequence in three series of tumours from three patients indicates a very low contamination of our samples with normal tissue.

Genetic changes in 9p21 and 17p13 are considered to be early and late events, respectively, in the development of bladder cancer [28]. Findings in most of our patients support this notion or are not at odds with it. Six patients carried a genetic alteration in 9p21 but not in 17p13 in their tumours, and earlier changes in 9p21 relative to 17p13 could be observed in two patients with suspected oligoclonality (see below). Four patients with genetic changes at both loci in all of their tumours and nine patients in whom no changes at all could be detected, yielded no information about the time order of the events. The remaining four patients harboured an alteration in 17p13 but retained heterozygosity for 9p21 markers in all tumours (three patients) or in a subset of tumours (one patient). Although early events in 17p13 cannot be excluded, heterozygous mutations or methylation silencing of the CDKN2A locus represent alternative explanations of these data.

The majority of our patients had concordant genetic findings (TP53 mutation and/or LOH patterns) in all of their tumours (18 of 25 patients, group A). TP53 mutations are unique genetic alterations, and their chance independent origin is very unlikely. Therefore, a repeated occurrence of the same TP53 mutation in recurrent or multifocal tumours of one patient is a very strong proof of monoclonality of these tumours. Further support for this mode of evolution of TCCs comes from patients without TP53 mutations but with identical LOH patterns in all tumours. There are only two alleles of each polymorphic DNA marker in patients constitutionally heterozygous for these markers, but repeated loss of one of these two alleles, especially in larger series of tumours and in many patients, is also unlikely to be a chance finding. Finally, taking into account the relatively high overall rate of genetic

alterations in the two regions studied (64% of patients, 52.3% of tumours), especially the large series of tumours from one patient which show absence of any detectable defect and retain heterozygosity in both regions, also support the notion that these tumours may in fact be genetically related.

The remaining seven patients had discordant findings in their tumours (group B). In five of these (patients 03, 06, 18, 21, 23), heterozygosity is observed in earlier tumours, which is replaced by LOH in a subset of later tumours. This pattern is consistent with parallel evolution and co-existence of the original tumour clone retaining heterozygosity, and possibly one divergent subclone derived from the original clone, which displays LOH. These two clones may alternately give rise to recurrent tumours. However, in the remaining two patients (group B, cases 15 and 25) the later tumour must have originated independently and could not be derived from the earlier tumour: the two tumours of patient 15 display loss of the opposite alleles, and the second tumour of patient 25 shows return from LOH to heterozygosity.

Two hypotheses have been proposed to describe the development of multiple and recurrent superficial bladder TCCs in one patient. Resuming data from several molecular studies, the monoclonal origin of the tumours is observed in the majority of cases. However, at the same time oligoclonality is found in a fraction of patients, thus providing molecular evidence for field cancerization [29]. Hartmann et al. [9] examined deletions on chromosome 9p, 9q and 17p in 52 superficial bladder TCCs from 10 patients and found four patients with an identical genetic alteration in all tumours, one patient with no genetic alteration, and five patients with two or more different clones in their tumours. Using 21 microsatellite markers on eight chromosomal arms, Takahashi et al. [30] found an identical microsatellite alteration pattern in 54% patients with upper urinary tract tumours and subsequent bladder tumours, and in 84% patients with recurrent bladder tumours. Hafner et al. [31] studied 14 informative patients with multifocal urothelial carcinomas of the upper and lower urinary tract, and found five patients (36%) with at least two tumour clones with different LOH patterns on chromosome 9 and 17p13. Generally, oligoclonality was more common in early lesions and it was suggested that monoclonality (or pseudomonoclonality) might be an effect of a massive growth of one tumour clone during the tumour progression [29]. Also in our study, the findings in 23 of 25 patients are compatible with the monoclonal model of recurrent and multifocal TCCs. Four of these 23 patients who carry identical TP53 mutations in all their tumours, and several other patients with identical LOH patterns in all their tumours offer a very strong support for monoclonality. However, the genetic constitution of two pairs of tumours in the remaining two patients is incompatible with monoclonality, and points to an independent origin of these tumours and to possible field cancerization.

In summary, our data indicate that at least in patients selected for high rate of recurrence of bladder cancer monoclonality is strongly predominating. This can have immediate practical diagnostic and therapeutic consequences. The identical genetic alterations in synchronous and metachronous lesions can be used for the monitoring of treatment efficacy and for screening of recurrences. The possible initiation of tumour recurrences by intraluminal seeding and implantation during TUR is a very strong argument for immediate postoperative intravesical chemotherapy instillation. A large meta-analysis of published results recently confirmed the effect of chemotherapy on a significant decrease of the risk of recurrences after TUR in patients with stage Ta or T1 single and multiple bladder cancer [32]. In monoclonal TCCs, the individual tumours show identical biological behaviour including their sensitivity or resistance to chemotherapeutic agents, and it allows the use of genetic markers in the evaluation of treatment efficacy [33]. Moreover, tumour cells with an identical molecular profile carry identical targets for perspective new types of specific targeted cancer therapy e.g. using antisense oligonucleotides.

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Research Article

Parallel optimization and genotyping of multiple single-nucleotide polymorphism markers by sample pooling approach using cycling-gradient CE with multiple injections

Increasing importance of single-nucleotide polymorphisms (SNPs) in determination of disease susceptibility or in prediction of therapy response brings attention of many molecular diagnostic laboratories to simple and low-cost SNP genotyping methodologies. We have recently introduced a mutation detection technique based on analysis of homo- and heteroduplex PCR fragments resolved in cycling temperature gradient conditions on a conventional multicapillary-array DNA sequencer. The main advantage of this technique is in its simplicity with no requirement for sample cleanup prior to the analysis. In this report we present a practical application of the technology for genotyping of SNP markers in two separate clinical projects resulting in a combined set of 44 markers screened in over 500 patients. Initially, a design of PCR primers and conditions was performed for each SNP marker. Then, optimization of CE running conditions (limited just to the proper selection of temperature cycling) was performed on pools of 20 DNA samples to increase the probability of having each of the two allele types represented in the sample. After selecting the optimum conditions, screening of markers in patients was performed using a multiple-injection approach for further acceleration of the sample throughput. The rate of successful optimization of experimental conditions without any pre-selection based on the SNP sequence or melting characteristics was 80% from the initial SNP marker candidates. By studying the failed markers, we attempt to identify critical factors enabling successful typing. The presented technique is very useful for low to medium sized SNP genotyping projects mostly applied in pharmacogenomic research as well as in clinical diagnostics. The main advantages include low cost, simple setup and validation of SNP markers.

Keywords: Cycling-gradient CE / Multiple injection / Prostate cancer / Schizophrenia / SNP
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1 Introduction

Genotyping of specific single-nucleotide polymorphisms (SNPs) is becoming increasingly important in the diagnosis of genetic risk factors for inherited disease susceptibility as well as in prediction of treatment response. The recent decade has seen a tremendous speed of development of various methodologies and instrumentation for SNP genotyping [1]. Among other parameters, increase in sample throughput has been the driving factor behind the new technology development. This is certainly the case in whole-genome SNP typing (and haplotyping) studies, where a vast number of potential marker candidates is screened in each sample. For such demanding applications, dedicated systems using massive parallel hybridization and direct readout are employed either in the classic microarray format [2], utilizing functionalized microbeads [3] or picotiter plates [4]. With resulting hundreds of thousands of genotypes obtained in each experiment, the ultrahigh-throughput platforms inherently

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Abbreviations: CGCE, cycling-gradient CE; ***Tm***, melting temperature

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require the ability to process enormous amounts of generated data. Therefore, such systems currently find their main use in pharmaceutical industry and large research centers capable to absorb the associated equipment cost. For common clinical diagnostics, disease risk assessment or therapy response prediction, the required throughput is usually lower as the number of individual polymorphisms with confirmed functional relevance and clinical outcome is still relatively low [5]. This opens a possibility for utilization of low to medium throughput technologies exhibiting sufficient capacity to deliver up to hundreds, rather than thousands, of assays per experiment.

Technologies for low- to medium-throughput SNP genotyping often employ tools and protocols commonly accepted in other molecular biology applications such as DNA sequence analysis or detection of somatic mutations. Experimental setup is usually based on existing instrumentation such as standard PAGE, UV/Vis or fluorescence spectrometers, PCR thermocyclers with or without real-time quantification capability, *etc*. Some of the techniques are based on DNA electromigration and performed with the help of CE as the separation platform. Since the attainment of the human genome, CE instrumentation has been routinely used in molecular biology. Capillary electrophoretic systems with 1, 8, 16 or 96 capillary arrays became routine inventory in many laboratories. Over the past years, their original mainstream use in DNA sequencing and microsatellite genotyping (mainly for human identification and paternity testing) has gradually been extended to other applications, most importantly in the area of discovery and screening of somatic mutations and typing of SNPs. Various adaptations of common gel electrophoresis methods and applications previously developed on home-built CE instruments have thus become routine on commercial systems. The most popular CE applications, other than sequencing or microsatellite genotyping, utilize fragment sizing by RFLP [6], amplified fragment length polymorphism (AFLP) [7], or following multiplexed ligation [8, 9] and allele separation by SSCP [10] or heteroduplex analysis [11]. A special group of methods employs separation due to partial DNA melting in CE-adapted versions of denaturing gradient gel electrophoresis techniques [12–14]. The universal applicability of CE instrumentation and simple transition among sequencing, genotyping and mutation detection applications is appealing to medium throughput environments such as core laboratories or diagnostic centers. The CE-based SNP typing methods usually exhibit a higher cost per single genotype in comparison to the previously mentioned ultrahigh capacity platforms; however, the main advantage is in their immediate availability and the low setup cost.

In 2003 we have originally introduced the cycling gradient CE (CGCE), sometimes also referred to as the cycling temperature CE, as a simplified CE version of classic temperature gradient gel electrophoresis with increased sample throughput [15]. The method is based on electrophoretic separation of homoduplex and heteroduplex DNA fragment forms under partial melting conditions in denaturing gel matrix. The two strands in each fragment are held together on one end by a 40 bp artificial high-temperature melting domain (GC clamp) attached to a 100–150 bp target sequence. The melting of the target sequence is achieved by applying periodical cycles of temporal temperature gradients. Similar to the other methods based on partial denaturation, the CGCE/cycling temperature CE technique exhibits superior resolution allowing to separate wild-type and mutant homoduplex and heteroduplex forms for any mutant appearing within the target sequence. The method has already been routinely applied to detect unknown somatic mutations from cancer tissue [16, 17].

In this work, we present further validation of the technique by optimization of experimental conditions with subsequent screening of indiscriminate sets of SNP markers in larger patient cohorts. Following optimization and elimination of unsuccessful markers from an initial group of marker candidates, we present screening of 13 SNPs in a homogeneous population of 85 patients diagnosed with schizophrenia and another 31 SNPs in a population of 487 prostate cancer patients and controls. In these two clinical projects we demonstrate the utilization of sample pooling in parallel assay optimization of multiple SNP markers [18] and utilization of multiple injection to increase sample throughput and to shorten the overall analysis times.

2 Materials and methods

2.1 Patients and SNP markers

The present work includes data from two separate projects aiming at inherited factors of disease predisposition. Blood samples from patients diagnosed with schizophrenia were obtained from the Center for Neuropsychiatric Studies at the Prague Psychiatric Center, and blood samples from a group of prostate cancer patients and controls with clinically confirmed benign prostatic hyperplasia were obtained from the Urological clinic, Faculty Hospital in Prague. Both projects were approved by the ethical committee of the 3rd Faculty of Medicine, Charles University, Prague.

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SNP markers studied in genetic predisposition of schizophrenia covered the following genes: *COMT*, *BDNF*, *RGS4*, *DTNBP*, *NRG1*, *DRD2*, *DRD3*, *HT2A*, *SERT* and *MAG*. For the prostate cancer study we have focused on selected polymorphisms in the *Androgene Receptor* gene as well as three other genes involved in testosterone synthetic pathway: *CYP17A1*, *SRD5A2* and *CYP3A4*.

2.2 DNA isolation and PCR amplification

Genomic DNA was isolated from patient blood samples using the GMC-ISOBL extraction kit (Genomac International, Prague, Czech Republic). All PCR reactions were performed using standard *Taq* polymerase (Qiagen, GmbH, Hilden, Germany), PCR primers were obtained from VBC-Genomics, GmbH (Vienna, Austria). Primers with GC-clamp extension were within 60–65 bp range, regular primers were within the 20–22 bp range. Analyzed PCR fragments were labeled with fluorescein. Standard PCR conditions with typical 35 cycles were applied. The PCR program was always concluded with a heteroduplex formation step consisting of denaturation at 95°C for 5 min, annealing at 65°C for 30 min and slow cooling to 4°C.

Theoretical melting temperatures (T_m) were calculated using WinMelt software (Medprobe, Oslo, Norway). In order to account for the effect of chemical denaturant present in the CE gel matrix, 21°C was deducted from the value obtained from WinMelt [19].

2.3 Cycling gradient CE

All CGCE experiments were performed on a standard MegaBACE™ 1000 96-capillary DNA analysis system (GE Healthcare Bio-Sciences, Piscataway, USA) using GMC-MB running buffer and GMC-MTRX denaturing separation polymer (Genomac International, Prague). Alternatively a standard long-read sequencing linear polyacrylamide (LPA) matrix (GE Healthcare Bio-Sciences) was used in some CGCE experiments resulting in identical resolution. During the optimization phase, temperature within the capillary array was monitored by a digital thermometer equipped with an external Pt-sensor. Cycling temperature profiles were programmed by MegaBACE temperature gradient creator (MBTG) version 2.0. (Genomac). For the screening phase, the MegaBACE was equipped with a robotic plate autoloader Caddy™ 1000 (Watrex Praha, Prague, Czech Republic), to allow for unattended automated overnight operation.

3 Results and discussion

The purpose of this work was to evaluate practical application of the CGCE technique in a typical clinical project. Unlike in other reports in which data is often presented only in a limited subset of optimized markers, the results presented here document real test of the approach on considerable sets of SNPs examined in large groups of patients. We choose to proceed methodically with no bias based on intermediate results. The processes of running the experiments and data evaluation were separated; the person executing the CE runs was blinded to the resulting data.

Both clinical projects reported here were directed at disease predisposition. Sequence information on all SNP markers was first obtained by searching literature and public databases. Using the sequence information surrounding the SNP position, the theoretical T_m of the fragment was calculated. To reflect the additional denaturing due to urea in the CE gel matrix, an experimentally determined constant of 21°C was deducted from the theoretical value. For each marker a target PCR segment was designed with an ideal length of 100–120 bp (not including the primer sites and GC-extension). The typical workflow is presented in Fig. 1. The optimization phase consisted of two steps: allele detection and subsequent validation. In the first step, 20 randomly selected patient DNA samples were mixed at equivalent concentrations. A set of PCR reactions, specific to each SNP marker, was then performed on the pooled sample [20]. The resulting PCR products were analyzed by CGCE using three temperature gradient programs consisting of 2 min cycles at $\pm$2°C around T_m-1, T_m and T_m+1, with T_m specific for each individual marker. Naturally, different SNP markers exhibiting the same T_m could be examined in parallel within the same 96-well plate, reducing the overall number of test runs required. After the first round of test runs, most markers already revealed characteristic temperature-dependent shifts when comparing positions of homo- and hetero-duplex peaks at different temperature gradient settings. In cases where the homo- and heteroduplex forms could not be identified, a set of the next 20 DNA samples was pooled, amplified and subjected to the same CGCE temperature gradient programs. Markers that did not show homo- and heteroduplex profiles after the first or second round (by which a total of 40 random samples has already been investigated) were assigned as failed. Since the migration order of individual duplex fragments given by the melting energies of homoduplexes can be directly translated into the base types (more stable G/C eluting initially and less stable A/T eluting later), it was possible at this point to directly assign genotypes. However, in order to exclude the

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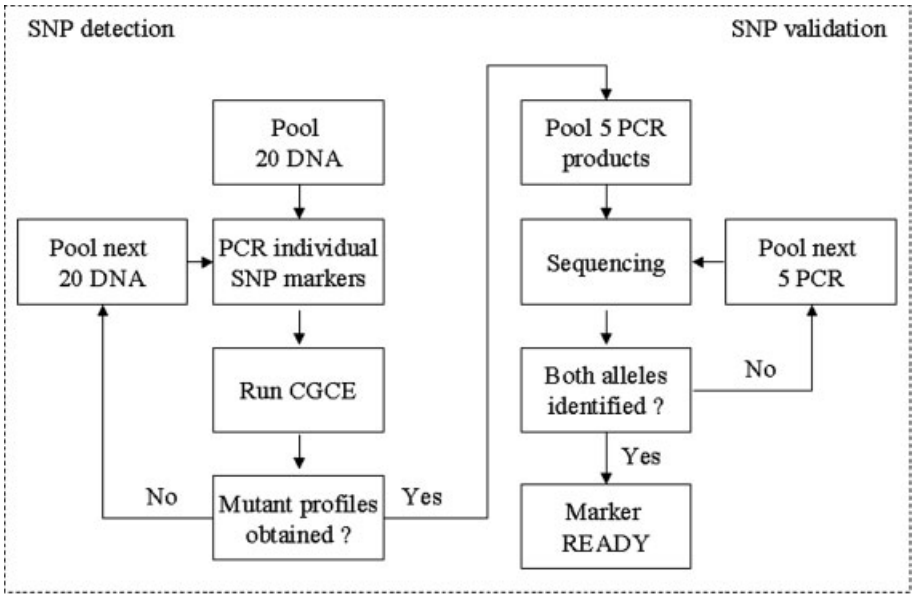

Figure 1. Scheme of SNP optimization and validation workflow.

possibility of a false positive due to the presence of another SNP at a different position within the same target sequence, identity of each marker was confirmed by sequencing. Since sequencing only reveals a minor allele if present in approximately 30–50% ratio, it was performed on PCR pools of at most five samples [21]. If no heterozygous position was detected a new pool of another five samples was taken.

From the initial group of 55 SNP candidates, the optimization phase produced positive results (unambiguous identification of all duplex forms) in 44 markers. This represents a success rate of 80%. All SNPs could be divided into four groups according to the quality of resulting peak profiles (see Fig. 2). The first group included markers that showed clearly separated peaks for all duplex forms and due to the distinct patterns their scoring could be done automatically by the software (Fig. 2A). A second group included markers whose heterozygous profiles did not always reveal all four duplex forms, but where the separated alleles could still be assigned with minimal intervention (Fig. 2B). The last group contained markers showing irregular profiles with asymmetrical peaks or additional system peaks (Fig. 2C); however, based on clear differences in peak patterns between homozygous and heterozygous control, genotypes could still be obtained after manual inspection and careful comparison of individual sample profiles. The remaining markers did not produce any conclusive peak patterns and therefore did not pass the CGCE test (Fig. 2D). Out of the 44 SNP markers passing the CGCE test, 24 (55%) were assigned as top quality, 12 (27%) as medium quality, and 8 (18%) as low quality. An overview of all SNP marker candidates analyzed in the present work is summarized in Table 1 with detailed information on marker type, allele frequency and resulting CGCE quality.

After the above described optimization phase was completed, SNP markers from A, B and C quality groups could finally be evaluated in large numbers of patient samples. During screening, each sample was first analyzed to distinguish homozygous status (only a single homoduplex peak in the electropherogram), heterozygous status (characteristic pattern of homo- and heteroduplex peaks) or to uncover failure of the PCR reaction (no peaks). If a homozygous status was detected, the sample was subsequently injected following heteroduplex formation with a PCR product of a known homozygous control. If the second analysis again resulted in a single homoduplex peak, the genotype of the tested sample was the same as the homozygous control. On the other hand, if the second analysis produced a characteristic heteroduplex pattern, then the inspected sample was assigned genotype opposite to the control.

In order to speed up the screening phase of the project, we decided to employ a multiple-injection technique. This technique, originally used to increase sample throughput of *MTHFR* mutation screening [22], takes advantage of the fact that during CE analysis, separating analytes usually occupy only a small portion of the entire capillary length. Consequently, the remaining open distance allows injecting a fresh sample mixture even before the separation of previously injected analytes is completed. Fragments from different injections are thus separated within individual non-overlapping regions in the capillary. We

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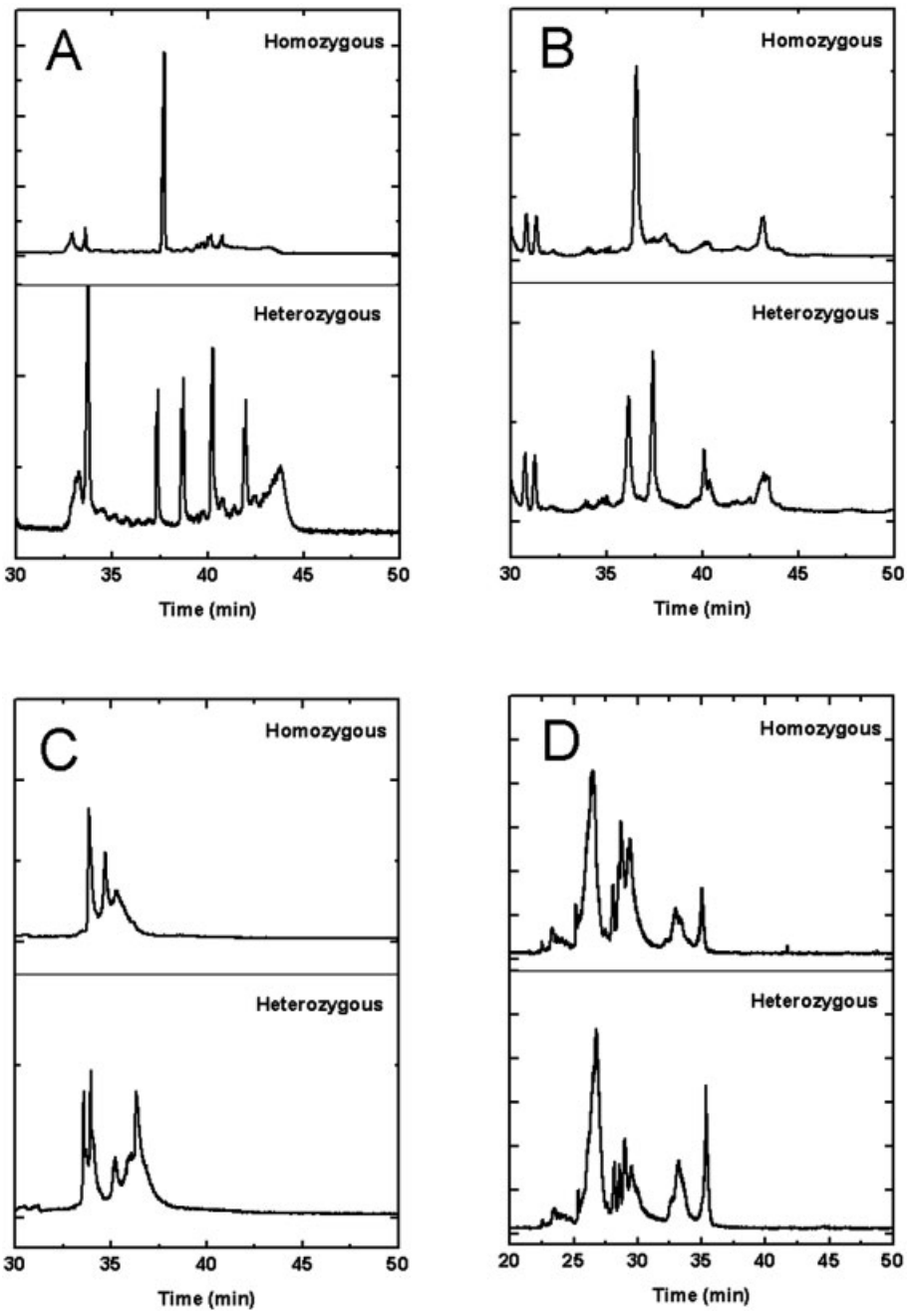

Figure 2. Examples of quality level types observed for CGCE separation of various SNP markers. (A) Top-quality markers suitable to automated scoring by software, (B) medium-quality markers capable of identification with minimum intervention, (C) low-quality markers requiring manual comparison of patterns, (D) failed markers which did not lead any information useable to allele scoring.

have previously illustrated application of this approach in CGCE for the detection of somatic mutations, where the repeated injections were carried out in synchronization with the temperature gradient cycles. The early as well as the late injected samples were thus subjected to the same temperature conditions [15]. In this work we have further refined this approach. Prior to each subsequent injection, the CE voltage was interrupted and the data collection was paused. Following the injection, data collection and the CE voltage were renewed. As a result, subsequent injections could be performed even while the previously introduced samples passed though the detector with no distortion of the resulting electropherograms. The number of possible injections performed within one analysis run was limited by the longevity of the separation gel matrix and the buffering capacity of the running buffer. In addition, periodical voltage interruptions, during which a longitudinal diffusion in the gel occurs, also affected the overall resolution by peak-broadening, especially if large numbers of injections were performed within a single run.

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Table 1. Overview of all SNP marker candidates evaluated in this study

Gene	SNP ID	Type	Major allele frequency	T_m (°C)	CGCE quality
Prostate cancer project					
CYP17A1	rs2486758	C/T	T 0.818	71	HIGH
	rs4919683	A/C	C 0.589	74	MEDIUM
	rs3740397	C/G	C 0.614	75	MEDIUM
	rs4919687	A/G	G 0.796	76	HIGH
	rs743575	A/C	A 0.789	75.4	HIGH
	rs3824755	C/G	G 0.732	76	HIGH
	rs284847	C/T	Not available	76	HIGH
	rs743572	A/G	A 0.562	77.4	MEDIUM
	rs6162	A/G	G 0.608	76.8	MEDIUM
	rs6163	A/C	C 0.562	73	LOW
AR[a)]	rs6152	A/G	G 0.762	78.5	–
	rs1337076	G/T	G 1.000	74	–
	SNP30	C/T	Not available	76.5	–
SRD5A2	rs2300697	C/T	T 0.622	65	MEDIUM
	rs4952219	A/G	G 0.598	62	HIGH
	rs12470143	C/T	C 0.533	69.2	HIGH
	rs2208532	A/G	A 0.594	66.4	MEDIUM
	rs2268796	A/G	G 0.542	69.8	LOW
	rs2300703	C/T	C 0.538	64.4	LOW
	rs4952220	A/C	A 0.541	64.6	MEDIUM
	rs612224	G/T	T 0.714	69	MEDIUM
	rs1042578	A/G	G 0.896	65	LOW
	SNP4	C/T	Not available	66.5	HIGH
	rs413836	A/G	G 0.750	–	–
	rs1475054	C/G	C 0.740	70	HIGH
	rs12712317	A/C	Not available	70.5	HIGH
	SNP17	A/G	Not available	73.4	HIGH
	rs676033	A/G	Not available	74.8	LOW
	rs11403363	T/–	Not available	–	–
CYP3A4	rs12333983	A/T	T 0.599	–	–
	rs2242480	A/G	G 0.639	72	–
	rs4646437	C/T	C 0.679	75.5	HIGH
	SNP15	C/G	Not available	68	–
	rs28988605	T/–	T 0.663	–	–
	rs1851426	C/T	C 0.869	74	HIGH
Schizophrenia project					
COMT	rs737865	C/T	T 0.792	73.2	MEDIUM
	rs4680	A/G	A 0.517	78	MEDIUM
	rs165599	A/G	Not available	78.5	LOW
BDNF	rs6265	A/G	G 0.825	73.5	HIGH
RGS4	rs10917670	C/T	Not available	68.2	HIGH
	rs951436	A/C	A 0.575	69.2	HIGH
	rs951439	C/T	C 0.517	71.4	HIGH
	rs2661319	C/T	Not available	75	HIGH
	rs10917672	C/T	Not available	75	HIGH
DTNBP	rs2619538	A/T	T 0.610	72.5	HIGH
	rs12204704	A/G	Not available	72.5	HIGH
	rs2743852	C/G	Not available	70.2	MEDIUM
	rs12525702	C/T	Not available	69	LOW
NRG1	SNP8NRG221533	C/T	Not available	62.5	HIGH
DRD 2	rs1801028	C/G	C 0.980	–	–
DRD 3	rs6280	C/T	T 0.650	77.2	HIGH
5-HT2A	rs6313	C/T	C 0.562	70.8	HIGH
SERT	rs4795541	N*	Not available	–	–
MAG	rs720308	A/G	A 0.844	74	MEDIUM
	rs720309	A/T	T 0.845	73.2	LOW

a) *Androgene Receptor* gene

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Therefore, under given experimental conditions, the maximum number of injections was set at four. Using this condition no notable distortion of resolution was observed. A typical result from such experiment is presented in Fig. 3 showing a four injection run with various SNP markers. In the presented case the alleles were assigned with the use of an internal heterozygous standard. An increase in one of the wild-type or mutant homoduplex peaks indicates a homozygous sample, symmetrical proportion of the two peaks indicates a heterozygous sample call.

An estimated gain in sample throughput using multiple-injection method can be determined using a simple calculation. For a given analysis during which the first fragments elute after a "dead" time of t_0, and where the time between the first and the last eluting peak is t_i, the total analysis time can be expressed as $t_0 + t_i$. In a standard series of multiple CE runs the total time can be expressed as $t_{total} = (t_0 + t_i) \times n$, where n is the number of runs. If multiple injections are performed, the total analysis time can be calculated as $t_{total} = t_0 + t_i \times n$ where n is the number of injections. Therefore, each consecutive injection increases the total analysis time only by the t_i., while in a conventional series of CE runs the time is increased by $t_0 + t_i$. In the present work, the first peak (usually a primer) typically appeared 25 min after the start and the last peak (usually a residual single-stranded fragment) eluted in approximately 45 min. Hence, for every subsequent injection the net gain in analysis time was approximately 25 min. In addition, time needed to replace gel matrix and run preconditioning [23] was also saved. Figure 4 demonstrates the difference in overall sample throughput between a normal series of CE runs and a series of multiple runs, each including four sample injections. The plot reflects the t_0 and t_i values, additional time required to perform each sampling within one multiple-injection experiment as well as setup time needed to replace gel matrix and to perform pre-conditioning prior to each CE run. It is clear that with the multiple injections the overall throughput is close to double.

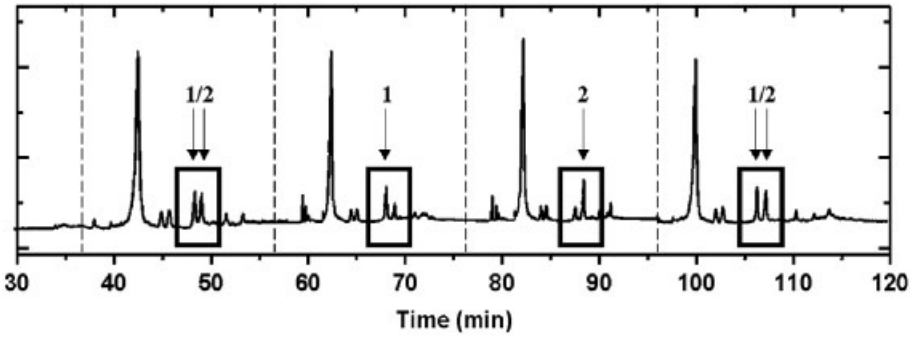

Figure 3. Genotyping of multiple SNP markers by CGCE with multiple injections using heterozygous internal control. The labels indicate allele calls.

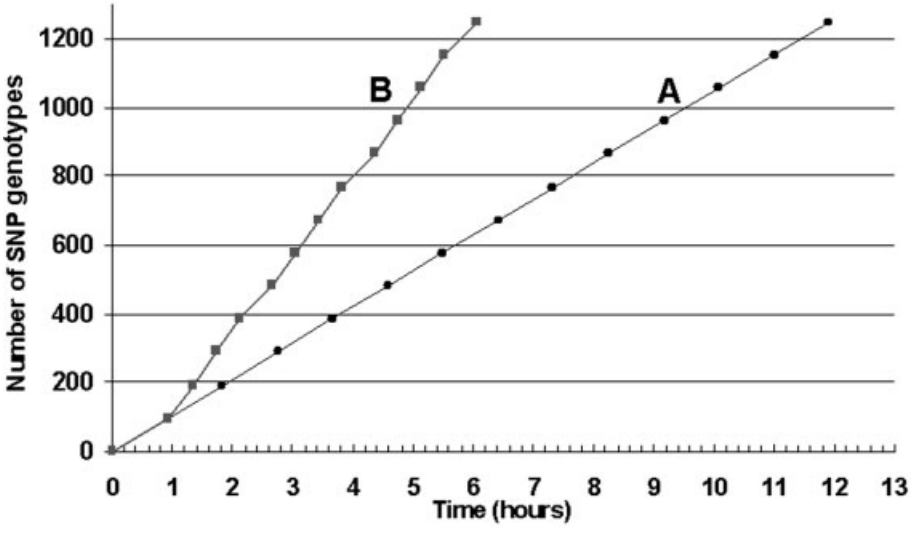

Figure 4. Effect of CE with multiple-injection approach to SNP screening sample throughput. (A) Processing time required for regular screening, and (B) time required for screening using four injections *per* analysis.

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4 Concluding remarks

Most current clinical applications of SNP genotyping are focused on investigating relatively small groups of 10–100 markers. Those include for example pharmacogenomic testing for targeted cancer therapy or genetic risk assessment in disease predisposition. In these cases, recently developed high-throughput SNP typing platforms producing thousands of genotypes per analysis are not optimal. Automated multicapillary DNA sequencers, widely introduced during the human genome project, are now standard in most molecular biology laboratories. These instruments are rarely running at full capacity, therefore there is a demand for new applications.

Sample pooling is an effective approach to create samples with both alleles represented on the resulting PCR product mixture. In the current study the overall rate for first-take successful optimization of experimental parameters in a set of 55 different SNP marker candidates was 80%. Identification of the factors behind the low quality is not straightforward. The poor performance of some markers is likely related to the secondary structure effects, hence, to the actual target sequence surrounding the SNP position. We expect that with an additional effort, primer design and experimental conditions could eventually be further evaluated to enable analysis of additional markers. Especially in cases where a functional relevance specific to a given polymorphism is key for subsequent clinical decisions, the effort in further optimization would be justified. The additional steps may include repositioning of the GC clamp from one primer to the other as suggested recently [17] or complete redesign of the PCR by repositioning the SNP position within the target sequence. Results of a separate subset of experiments (data not shown) suggest that other techniques utilizing partial melting, such as dHPLC or denaturing gradient gel electrophoresis, are also likely to fail in case of the markers that did not lead positive results in CGCE testing. In those instances a method based on a principle other than denaturation should be used, such as selective restriction (RFLP) or allele-specific PCR. By using multiple-injection in each run, we have dramatically shortened the overall time required to screen multiple SNPs in hundreds of samples. Further improvement in sample throughput and level of automation in allele calling can be achieved by using alternative fluorescent tags for labeling of the tested sample and the internal standard or even to better distinguish individual strands within the same PCR fragment.

The overall feasibility of the technique has been presented on two separate clinical projects scrutinizing the role of genetic polymorphisms in disease predisposition. We have optimized 13 SNP markers and performed screening of a homogeneous population of 85 individuals with clinical diagnosis of schizophrenia. In addition, we have succeeded in finding optimum conditions for analysis of another 31 SNPs and genotyped a group of 274 prostate cancer patients and 213 controls with conformed benign prostatic hyperplasia. Clinical outcome of the two projects will be discussed in separate reports.

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Frekvence výskytu vrozených DNA polymorfismů genů syntetické dráhy testosteronu v České populaci pacientů s karcinomem prostaty

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Klíčová slova: karcinom prostaty, DNA, polymorfismy, genotypování, CYP17A1, CYP3A4, SRD5A2

Cíl: Růst prostatických buněk závisí na hladině aktivního testosteronu, který je tak již v nízkých dávkách významným tumor promotorem. Sledování aktivity genů zapojených do regulace syntézy testosteronu má proto význam pro výzkum mechanismu vzniku a proliferace nádorových buněk prostaty. Hlavním záměrem prezentované studie bylo zjištění výskytu a frekvencí nejvýznamnějších jednonukleotidových polymorfismů skupiny genů syntetické dráhy testosteronu v české populaci pacientů s karcinomem prostaty.

Metodika: Do studie bylo zařazeno celkem 237 pacientů s karcinomem prostaty a 229 kontrolních osob s klinicky ověřenou diagnózou benigní hyperplázie. U každé osoby bylo provedeno vyšetření sady 30 jednonukleotidových polymorfismů z DNA získané z periferních lymfocytů.

Výsledky: Získané frekvence výskytu polymorfních alel se mezi skupinou pacientů s karcinomem a pacientů s benigní hyperplázií u většiny markerů výrazně neliší. Nadějným výsledkem je však identifikace markeru SR-49B (dbSNP ID rs4952219) v genu SRD5A2 u kterého byl zjištěn statisticky významný rozdíl poměrů jednotlivých alel i jednotlivých genotypů při srovnání sledované a kontrolní skupiny.

Diskuse: Polymorfismus SR-49B má potenciál pro odhad vrozené predispozice výskytu karcinomu prostaty. Žádný s ostatních sledovaných markerů takovou asociaci nevykazuje.

1. Úvod

Karcinom prostaty (KP) je na druhém místě v incidenci nádorových onemocnění u mužů. Populační incidence nádoru prostaty vykazuje závislost na celé řadě faktorů. Mezi hlavními jsou rasa, věk a rodinná historie tohoto onemocnění. Je dokázáno, že zatímco afro-americká populace vykazuje téměř dvakrát vyšší incidenci ve srovnání s evropskou populací, nejnižší výskyt je u Asiatů. Uvedené rasové předpoklady mohou naznačovat genetické predispozice, avšak i ty lze interpretovat jako působení vnějších karcinogenních promotorů podle geografické lokace nebo rozdíly volby medicínských postupů. Nejvyšší incidence karcinomu prostaty v Evropě je ve Finsku – 101/100 000 mužů a je například 4x vyšší než v Řecku, kde je 24/100 000 mužů. Čísla v ČR se pohybují někde uprostřed těchto krajních hodnot. V roce 2003 byla incidence karcinomu prostaty 74,9/100 000 mužů a mortalita 27,0/100 000 mužů. Ve státech Evropské unie je ročně diagnostikováno 85 000 nových případů karcinomu prostaty, v České republice pak přibližně 2500 případů ročně. Závislost na věku naznačuje významnost dlouhé exponovanosti endogenními (hormony) nebo exogenními faktory (vnější karcinogeny). Padesátiletý muž se životním výhledem 25 let má riziko mikroskopického nálezu karcinomu prostaty 30 %, klinicky signifikantního nálezu 10 % a riziko úmrtí na

karcinom prostaty 3 %. Nejnovější epidemiologické údaje svědčí o tom, že muž s lokalizovaným a dobře diferencovaným karcinomem prostaty má 9% riziko úmrtí na tento karcinom do 15 let. Přibližně třetina pacientů má stanovenu diagnózu v době lokalizovaného onemocnění, necelých 20% pak v době generalizace. Poslední významný faktor tohoto onemocnění – rodinná anamnéza jasně ukazuje na genetickou predispozici. Nedávné studie teorii existence těchto genetických predispozic jednoznačně podporují.

Je známo, že růst prostatických buněk závisí na hladině aktivního testosteronu. Vzhledem k tomu, že u myší může být adenokarcinom prostaty vyvolán silnými dávkami testosteronu, je pravděpodobné, že testosteron je již v nízkých dávkách významným tumor promotorem. Studie popisující vysoké hladiny testosteronu u pacientů s nádorem prostaty jsou výjimečné, naopak nízké hladiny testosteronu, indikující androgenovou nezávislost, byly označeny za produkt velmi agresivních forem této nemoci a celkově horší prognózu u pokročilých stadií. Zdá se tedy pravděpodobné, že nikoli vysoké hladiny testosteronu, ale především dlouhodobá expozice testosteronu způsobuje predispozici ke vzniku nádoru prostaty. Ve shodě s touto hypotézou je i to, že se nádor objevuje především ve vyšším věku a že toto onemocnění nepostihuje muže kastrované v mladém věku. Z popsaných důvodů jsou geny mající roli při syntéze testosteronu potenciálními kandidáty při sledování molekulárně-genetického mechanismu růstu nádorových buněk prostaty (1).

Jednonukleotidové polymorfismy (SNP) jsou jednoduché záměny (substituce) nukleotidů v DNA sekvenci, které lze vypozorovat v průměru každých 200 – 300 bází. Nacházejí se v kódujících (exonech) i nekódujících (intronech) částech genů a představují nejčastější formu genových variací. Na rozdíl od vrozených bodových mutací, u kterých lze ze záměny báze vysledovat přímou spojitost se změnou funkce proteinu, funkční důsledek SNP často nelze přímo identifikovat. Vzhledem k jejich častému výskytu v regulačních regionech jako jsou například místa vázání transkripčních faktorů, se jejich působení často prezentuje jako ovlivňování genové exprese. Z tohoto důvodu je jim také připisována hlavní role v dědičnosti při rozdílech v genetické predispozici onemocnění, včetně nádorů prostaty(2).

Předkládaná studie měla za cíl screening SNP polymorfismů u pacientů s nádorem prostaty pro nalezení vztahů s genetickou predispozicí u tohoto onemocnění. Studie byla zaměřena na skupinu vybraných 30ti polymorfismů v genech syntetické dráhy testosteronu CYP17A1, CYP3A4 a SRD5A2 (3,4,5).

2. Materiál a metodika

2.1. Pacienti

Do studie bylo v zařazeno celkem 237 pacientů ve věku od 50 do 73 let s histopatologicky ověřeným karcinomem prostaty diagnostikovaných a léčených na Urologické klinice 3. LF UK a FNKV. Do souboru byli zařazeni pacienti s anamnesticky i nově diagnostikovaným (ověřeným) karcinomem prostaty. Verifikace byla prováděna s použitím digitálního rektálního vyšetření, hodnoty prostatického specifického antigenu (PSA), transrektálního sonografického vyšetření a histopatologického vyhodnocení biopsie prostaty, preparátu z radikální prostatektomie nebo transuretrální resekce prostaty. Současně byla vyhodnocena agresivita nádoru (Gleason score). Kontrolní skupinu tvořilo 229 pacientů s negativním nálezem. Negativita byla prokázána nízkou hodnotou věkově specifického PSA společně s negativním digitálním rektálním

vyšetřením. Současně byla vždy také potvrzena histopatologickým nálezem benigní hyperplazie z preparátu transvezikální prostatektomie či transuretrální resekce prostaty.

Pacientům i kontrolám bylo odebíráno 10 ml nesrážlivé krve a zasláno na molekulárně-genetické vyšetření.

2.2. Molekulárně-genetický screening DNA polymorfismů

Izolace DNA z krevních vzorků byla prováděna standardní metodou extrakce na kolonkách za použití komerčního kitu GMC blood DNA isolation kit (Genomac International, ČR). Sledované polymorfismy byly vytipovány na základě výběru ve veřejně přístupných SNP databází GeneSNPs, HGMD, dbSNP a HGVbase. Amplifikace úseků obsahujících sledované polymorfismy (typicky o délce 100 – 140 bází) byla provedena s použitím podmínek specifických pro danou sekvenční oblast. Při amplifikaci bylo použito fluorescenční značení jednoho s použitých primerů. Po reakci byly fluorescenčně značené amplifikáty analyzovány v multikapilárním genetickém analyzátoru. Screening byl prováděn s použitím techniky kapilární elektroforézy v cyklujícím teplotním gradientu (Cycling Gradient Capillary Electrophoresis, CGCE). Tato metoda je založena na principu separace heteroduplexů za podmínek jejich částečné denaturace (6). Z úvodní skupiny cca 85 kandidátů byla provedena selekce konečných 30ti markerů na základě optimalizace podmínek PCR a CGCE. Detailní popis metodiky optimalizace jednotlivých markerů a následného screeningu krevních vzorků sledované a kontrolní skupiny byl publikován separátně (7).

2.3. Statistické hodnocení

Statistická významnost rozdělení alel a rozdělení genotypů u sledované a kontrolní skupiny byla zjišťována s použitím neparametrického χ^2 testu na 5% hladině významnosti.

3. Výsledky

Bylo provedeno vyšetření 30 markerů u skupiny 237 pacientu s diagnózou karcinomu prostaty a 229 kontrolních subjektů s nálezem benigní hyperplázie prostaty, úhrnem se tedy jednalo o 13980 genotypizačních vyšetření. U každého sledovaného polymorfního markeru byla vypracována distribuce alel pro skupinu karcinomů a pro kontrolní skupinu. Současně byla v databázích vyhledána udávaná průměrná distribuce pro evropskou/kavkazskou populaci. Výsledky jsou shrnuty v Tabulce I A až C.

Tabulka I. Rozdělení frekvence alel sledovaných polymorfismů. Uváděné hodnoty odpovídají skupině pacientů s karcinomem (KP), kontrolní skupině benigních hyperplázií (BHP) a obecnému rozdělení alel v evropskou/kavkazské populaci dle veřejných databází (DB).

A – polymorfismy genu CYP17A1

CY-248 rs2486758

	T	C
KP	0,778	0,222
BHP	0,802	0,198
DB	0,750	0,250

CY-491 rs4919683

	A	C
KP	0,435	0,565
BHP	0,441	0,559
DB	0,400	0,600

CY-S3 rs3740397

	G	C
KP	0,407	0,593
BHP	0,396	0,604
DB	0,392	0,608

CY-S6 rs4919687

	A	G
KP	0,268	0,732
BHP	0,299	0,701
DB	0,331	0,669

CY-S7 rs743575

	C	A
KP	0,269	0,731
BHP	0,309	0,691
DB	0,333	0,667

CY-S8 rs3824755

	C	G
KP	0,147	0,853
BHP	0,130	0,870
DB	0,104	0,896

CY-S8B rs284847

	A	G
KP	0,043	0,957
BHP	0,033	0,967
DB	0,025	0,975

CY-S29 rs743572

	G	A
KP	0,403	0,597
BHP	0,401	0,599
DB	0,400	0,600

CY-S31 rs6162

	A	G
KP	0,424	0,576
BHP	0,454	0,546
DB	0,408	0,592

CY-S32 rs6163

	A	C
KP	0,393	0,607
BHP	0,422	0,575
DB	0,392	0,608

B – polymorfismy genu CYP3A4

CP-S5 rs12333983

	A	T
KP	0,094	0,906
BHP	0,074	0,926
DB	0,117	0,883

CP-S13 rs4646437

	T	C
KP	0,096	0,904
BHP	0,083	0,917
DB	0,133	0,867

CP-S47 rs1851426

	T	C
KP	0,032	0,968
BHP	0,039	0,961
DB	0,042	0,958

C – polymorfismy genu SRD5A2

SR-49B rs4952219

	A	G
KP	0,393	0,607
BHP	0,467	0,533
DB	0,625	0,375

SR-124 rs12470143

	T	C
KP	0,466	0,534
BHP	0,491	0,509
DB	0,6250,625	0,375

SR-220 rs2208532

	G	A
KP	0,478	0,522
BHP	0,404	0,596
DB	0,342	0,658

SR-236 rs2365778

	T	C
KP	0,532	0,468
BHP	0,489	0,511
DB	0,625	0,675

SR-275 rs2754530

	T	C
KP	0,331	0,669
BHP	0,317	0,683
DB	0,792	0,208

SR283A rs28383003

	G	T
KP	0,307	0,693
BHP	0,304	0,696
DB	0,318	0,682

SR-283B rs28383002

	T	A
KP	0,307	0,693
BHP	0,304	0,696
DB	0,318	0,682

SR-495 rs4952220

	C	A
KP	0,429	0,571
BHP	0,459	0,541
DB	0,275	0,725

SR-612 rs612224

	G	T
KP	0,331	0,669
BHP	0,328	0,672
DB	0,200	0,800

SR-S1 rs1042578

	A	G
KP	0,108	0,892
BHP	0,162	0,838
DB	0,068	0,932

SR-S4 nenalezen

	T	C
KP	0,990	0,010
BHP	0,861	0,139
DB	neuvedeno	neuvedeno

SR-S12 rs413836

	A	G
KP	0,510	0,490
BHP	0,388	0,612
DB	0,250	0,750

SR-S15A rs1475054

	G	C
KP	0,328	0,672
BHP	0,236	0,764
DB	0,15	0,85

SR-S15B rs12712317

	A	C
KP	0,010	0,990
BHP	0,125	0,875
DB	0,036	0,964

SR-S17 nenalezen

	A	G
KP	0,461	0,539
BHP	0,435	0,565
DB	neuvedeno	neuvedeno

SR-S26 rs676033

	A	G
KP	0,309	0,691
BHP	0,298	0,702
DB	0,200	0,800

SR-23 rs2300697

	C	T
KP	0,477	0,523
BHP	0,400	0,600
DB	0,333	0,667

4. Diskuse

Studium aktivity genů účastnících se systému testosteronové syntézy a odbourávání je již řadu let v popředí zájmu výzkumu mechanismů vzniku a progrese karcinomu prostaty. Prvním z genů, na který byl v této studii výběr zaměřen je gen z rodiny Cytochrom P450 označovaný jako CYP17A1 kódující steroid 17-alfa-hydroxylázu, který svojí lyázovou aktivitou umožňuje syntézu testosteronu ve varlatech (8). V tomto genu bylo vyšetřováno celkem 10 polymorfních markerů (viz Tabulka IA). Při srovnání frekvencí alel skupiny karcinomů a kontrolní skupiny nebyla ani v jednou případě odhalena významná odchylka, poměry alel odpovídají průměrnému rozdělení pro evropskou/kavkazskou populaci uváděném v mezinárodních databázích.

Dalším genem byl Cytochrom P450 3A4, CYP3A4, který je jedním ze základních metabolických faktorů účastnící se řady významných metabolických drah, včetně androgenní syntézy a odbourávání testosteronu (9). Zde byly vyšetřovány 3 polymorfismy (viz Tabulka IB). Ani v tomto případě nebyla u žádného ze sledovaných polymorfismů vysledována statisticky významná odchylka rozdělení alel skupiny karcinomů oproti kontrolní skupině BHP. U jednoho CYP3A4 polymorfismů byl zjištěn statisticky významný rozdíl v poměrech jednotlivých alel oproti průměrným hodnotám získaným z databáze. U markeru CP-S5 (dbSNP ID rs12333983) byl zjištěn poměr alel A/T u kontrolní skupiny 10%/90%, zatímco poměr uváděný v databázích pro evropskou/kavkazskou populaci je 15%/85%. Rozdíl je statisticky významný ($\chi^2 = 5,31$, $p \leq 0,025$) a může vyjadřovat specificitu zvolené kontrolní populace pacientů s diagnózou benigní hyperplázie. Vzhledem k relativně nízkému počtu vzorků analyzovaných pro zápis do mezinárodní databáze (udávaná hodnota 110 genotypů) však z tohoto nelze usuzovat přímo na roli tohoto markeru v genetické predispozici BHP.

Poslední ze sledovaných genů byl gen pro steroidní 5-alfa reduktázu (SRD5A2), který katalyzuje konverzi testosteronu na aktivní formu dihydrotestosteron (10). V tomto genu bylo vyšetřeno celkem 17 polymorfních markerů. U markerù SR-23 (dbSNP ID rs2300697) byl poměr alel C/T u karcinomů 48%/52%, zatímco tabulková hodnota populačního rozdělení z databáze je 33%/66%. Rozdíl je statisticky významný (χ^2 = 6,69, p ≤ 0,01), avšak vzhledem k tomu, že při přímém srovnání karcinomů a kontrol získaných v této studii významný rozdíl nalezen nebyl (χ^2 = 2,66, p ≤ 0,20), lze shora uvedené zjištění i v tomto případě považovat za odchylku použité populace BHP pacientů oproti průměrné normální populaci uvedené v databázi. I v tomto případě by pro prokázání genetické predispozice BHP bylo třeba provést genotypování více vzorků normální populace.

Patrně nejzajímavějším výsledkem předkládané studie je však výsledek pro SRD5A2 marker SR-49B (dbSNP ID rs4952219). Poměr alel A/G u karcinomů byl 39%/61%, což je statistický významně odlišné od poměru alel získaného pro kontrolní skupinu 47%/53% (χ^2 = 5,33, p ≤ 0,025). U tohoto markeru bylo následně provedeno i srovnání výskytu jednotlivých genotypů. U skupiny karcinomů to bylo 38 homozygotů AA, 111 heterozygotů AG a 88 homozygotů GG, zatímco u kontrolní skupiny to bylo 61 homozygotů AA, 93 heterozygotů AG a 76 homozygotů GG. I pro rozdělení genotypů byl u tohoto polymorfismu zjištěn statisticky významný rozdíl (χ^2 = 7,71, p ≤ 0,025).

5. Závěr

V prezentovaném projektu bylo provedeno vyšetření celkem 30 DNA polymorfismů v genech syntetické dráhy testosteronu. Při srovnání skupiny 273 pacientů s karcinomem a 229 pacientů s nálezem benigní hyperplazie nebyl u naprosté většiny markerů nalezen významný rozdíl v poměru příslušných alel. Statisticky významné rozdíly poměrů alel u markerů CP-S5 (dbSNP ID rs12333983) v genu CYP3A4 a SR-23 (dbSNP ID rs2300697) při použití údajů normální populace z databáze lze připisovat volbě kontrolní skupiny pacientů s benigní dysplázií, případně také relativně nízkému počtu genotypů použitých pro údaje v mezinárodní databázi.

U markeru označeného jako SR-49B (dbSNP ID rs4952219) v genu SRD5A2 je zjištěný signifikantní rozdíl poměru alel i poměru jednotlivých genotypů významným výsledkem indikujícím jeho potenciální asociaci s predispozicí ke karcinomu prostaty. Vzhledem k tomu, že tento marker zatím nebyl v literatuře v této souvislosti popsán, bylo by pro potvrzení třeba provést další vyhodnocení, především za použití rozboru dědičnosti jednotlivých genotypů u příbuzných pacientů s karcinomem.

6. Poděkování

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Genetic variations in epidermal growth factor receptor gene (EGFR) and their role in prediction of response to gefitinib therapy in non-small cell lung cancer

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Summary: Background: Somatic mutations in tyrosin-kinase domain of EGFR gene were found as valuable predictors of clinical response in gefitinib treated non-small cell lung cancer (NSCLC) patients. The aim of this study was to investigate types and frequencies of EGFR mutations in Czech population of gefitinib responders with adenocarcinomas and bronchioloalveolar carcinomas (BAC).

Material and methods: Only patients with adenocarcinomas and BAC treated with gefitinib for at least 6 months and showing clear regression or stabilization were enrolled into the study. Somatic mutations were investigated from paraffin-embedded tissue and cytology smears by standard sequencing of four amplified exons (18-21), which encode tyrosin-kinase domain of the EGFR gene.

Results: Based on the selection criteria a total of 23 patients was in the studies group, 12 men and 11 women, all ranging from 34 - 77 years of age with the mean age of 65 years. EGFR mutations were found in 13 patients, 8 patients did not carry any detectable EGFR mutations and samples collected from the remaining 2 patients failed to yield sufficient amounts of DNA. Most frequent type of mutation was deletion in exon 19 (13 patients), which was found also in combination with other mutations. Less frequent were point mutations in exon 18 (two patients) and in exon 20 (one patient). Median survival times in responders with or without EGFR mutations were 440 days vs 326 days. When comparing to the original group of treated patients (responders + non-responders), mutations were found in 8 out of 39 female adenocarcinoma patients (20%) and in 5 out of 50 adenocarcinoma male patients (10%).

Conclusion: Somatic mutations of EGFR gene are significant in prediction of successful targeted biological therapy. The most frequent type is deletion in exon 19. Female adenocarcinoma patients bearing EGFR mutations are the most likely to profit from the therapy. For other patient subgroups, other prognostic markers are likely to be needed.

Keywords: EGFR, DNA, Mutations, Gefitinib, NSCLC, Treatment

Introduction:

Lung cancer is a serious civilization disease with high incidence and mortality. Despite to the booming development of new drugs and alternative treatment schemes the prognosis remains very poor, mean survival rate of advanced stages is only 6 - 8 months from the time of diagnosis, 5-year survival rate is only 5,5% for men and 7,3% for women. This puts mortality rates at the level of incidence rates (1).

Non-small cell lung cancer (NSCLC) representing about 80% of all lung cancers exhibits generally worse response to chemotherapy. Most patients with NSCLC in stage IIIB and IV are treated with combined chemotherapy. Aside from the conventional approaches, targeted biological therapy by epidermal growth

factor receptor inhibitors represents a very promising new modality (2-6). Over the past several years, gefitinib and erlotinib have proven highly effective in a number of heavily pretreated NSCLC patients. Even in cases where patients failed to respond to chemotherapy, the targeted biological therapy resulted in an increase of life expectancy as well as the quality of life. Although response to biological therapy is apparent, the overall statistics of patients having objective tumor response from this type of treatment seems relatively low. The average numbers range from 8.5 to 18.4%, depending on the race of the studied cohort, while disease control rates are between 42.2 and 54.4% (4,6). Counterbalancing the setback from overall low response rates is the existence of a number of clinical factors, such as smoking status, tumor subtype and gender that can be applied to predict the outcome of the treatment (7,8).

Research and investigation of modern therapy approaches in clinical oncology is directed towards finding predictors of treatment response at molecular levels (2,9-12). The molecular biomarkers often include somatic gene variations. Unlike inherited nucleotide polymorphisms, which are present in every cell in the human body, somatic gene variations represent molecular fingerprint specific to the tumor. They arise from transformation processes within the neoplastic cells and, therefore, can be detected in tissue samples from biopsies or resections. The most common types are somatic base substitutions (i.e. point mutations) followed by allelic deletions, hypermethylation, gene amplification and overexpression (13). In case of lung cancer, recent studies have demonstrated that presence of somatic gene variations may indicate positive response to targeted biological therapy. Among the various factors associated with better response, occurrence of mutations within the tyrosin-kinase domain of EGFR gene has been most frequently observed (10,11).

The aim of the presented study was to evaluate occurrence of EGFR mutations in a group of NSCLC patients who have previously shown regression or stabilization following gefitinib therapy. The main goal was to identify the most frequent mutations and their predictive value by correlation to several clinical factors.

Patients and Methods:

The selection of patients was based on clinical results from the gefitinib Early Access Program (EAP), including a total of 241 individuals treated at the Department of Pneumology. Only patients with adenocarcinomas and bronchioloalveolar carcinomas (BAC) showing a clear regression or stabilization within at least 6 months of gefitinib therapy were selected for this study.

Tumor tissue samples were collected during bronchoscopy examination and processed into either formaline-fixed paraffin-embedded sections or cytological slides. The total number of examined samples represented 15 paraffin sections and 15 cytology slides as multiple samples were obtained from some patients. The location of tumor cells within the sample was marked prior to DNA extraction.

Genomic DNA for mutation analysis was extracted by standard column extraction method using JetQuick isolation kit (Genomed, G.m.b.H, Loehne, Germany). Presence of somatic EGFR gene mutations was examined in exons 18, 19, 20 and 21, which encode for the tyrosin-kinase domain. Extracted DNA was first subjected to PCR amplification with PCR primers specific for each of the examined exons. The primer sequences were taken from ref. (10). In order to increase mutation detection sensitivity, deletions in exon 19 were detected by fragment analysis as described in a separate report (ref). All other exons were examined by direct sequencing of amplified PCR fragments using standard DYEnamic terminator sequencing kit (Amersham Biosciences, Piscataway, NJ). Fragment analysis and sequencing was performed on a 96-

capillary array sequencer (MegaBACE 1000, Amersham) equipped with Caddy 1000 robotic plate loader (Watrex Praha, Prague, Czech Republic) for unattended automated operation.

Results:

Of the initial 241 patients treated at our department during the gefitinib Early Access Program, 52 patients received gefitinib for at least 6 months. From this group 23 responders with adenocarcinoma and BAC were enrolled into this study. The group consisted of 11 females and 12 males, ranging from 34 to 77 years of age, with a mean age of 65.1 years.

Genomic DNA was successfully isolated from 160 out of 185 total samples (86,4% success). Of that, success rate was slightly better for fixed-tissue slices (90 out of 81, 90%) compared to cytology slides (79 out of 95, 83%). Somatic EGFR mutations were found in 13 patients (62%) of whom 8 were women (8 out of 11, 72.7%) and 5 were men (5 out of 12, 41.6%). All of mutation-positive samples displayed multiple base deletions in exon 19. In 3 of those cases mutation in other exon was found in addition to the exon 19 deletion. The other mutations were found in exons 18 (2 cases, Figure 1) and 20 (1 case, Figure 2, marked with "M"). No mutation was detected in exon 21. An overview of detected mutations is shown in Table I. The mean survival time for responders with EGFR mutation was 440 days versus 326 days in responders with no detectable mutation. In addition to the somatic mutations (occurring solely in tumor tissue) 14 patients displayed A/G or G/G (non-wildtype) genotype of an inherited single-nucleotide polymorphism found in exon 20, dbSNP reference# rs10251977). (Figure 2, marked with "P").

Discussion:

The initial papers describing occurrence of EGFR mutations in NSCLC have demonstrated strong association with positive response to gefitinib therapy, especially in patients with adenocarcinoma and BAC. The mutation frequency in Japanese population was 25% (15 out of 58) in one report (11) and 40% (111 out of 277) in another (12). Frequency reported for Caucasian populations was lower, typically between 8 and 15% (10,14). The median survival time in mutation positive responders exceeded 18 months, while no mutation was been found in patients non-responding to the therapy. In overall, mutations were more frequent in females, adenocarcinomas and in non-smokers.

The results of the Iressa compassionate use (Expanded Access) program in Czech Republic are in agreement to similar studies conducted in other countries. The overall positive response rate is usually not more than 10%, mostly in patients with adenocarcinomas and bronchioloalveolar carcinomas (7,8). The genotypic selection is very likely the main cause of the seemingly low overall response rates. The EGFR mutations were examined in two types of archived tissue samples, cytology slides and paraffin sections. The efficiency of DNA extraction followed by PCR amplification was 100% in cytology smears (15 out of 15) but only 60% in paraffin embedded tissue (9 out of 15). The main cause of failed DNA extraction was apparently the low quality of the paraffin sections. The failure to yield a good PCR amplification product could mostly be attributed to a known effect of DNA fragmentation within the formaline-fixed paraffin tissue (15,16). It is likely that the success rates would improve with better quality of the archived biological material or by preferring cytology smears.

A separate factor, affecting the success rates of positive sample detection is the sensitivity of mutation detection method. The inherent background coming from the normal un-mutated cells always limits detecting mutations in tissue samples. With the conventional sequencing approach it is likely, that some mutations pass undetected (14). Alternative approaches should therefore be evaluated, including targeted tumor cell selection by microdissection techniques (17) and high-sensitivity mutation detection methods (18).

The most frequently mutation type was deletion in exon 19. This is in agreement with results published by others (10-12,14). These findings suggest exon 19 having the primary importance supplemented with significantly less frequent alterations in other exons of the EGFR tyrosin-kinase domain in our population. Somewhat surprising was the absence of detectable mutations in exon 21, which is commonly perceived as moderately frequent. Clearly a larger cohort would be needed to capture other, lower frequency mutations. Importance the inherited polymorphism in exon 20 is currently not known. Further investigation will be necessary in order to evaluate its possible involvement in cancer susceptibility or association with success rates for treatment.

The above results indicate ability to predict outcome of long-term treatment in at least 72% of gefitinib responding women (8 out of 11) and 41.6% of men (5 out of 12). It is therefore important to search for additional molecular predictors that would complement the EGFR mutations. For example, amplification and overexpression of EGFR gene appears potentially very valuable (9,19). In addition, other members of the tyrosin-kinase receptor family, including HER-2 (ERBB2) and HER-3 (ERBB3) are also now under inspection (13). A combination of several predictors may offer improvement in assessment the expected outcome of the treatment not only in adenocarcinomas, where the biological therapy seems beneficial, but also in other types of lung carcinomas, where EGFR mutations are less frequent (2). EGFR amplification should be better predictor of stabilization rather than tumor regression (9).

Conclusion:

Our results confirm high frequency of somatic EGFR mutations in patients responding to gefitinib therapy. Therefore, no further profit is to be expected from large randomized chemotherapy studies without employing genetic predictors. The most proper predictor for our population seems detection of deletions in exon 19 of the EGFR tyrosin-kinase domain. In order to detect mutations in small populations of cancerous cells, alternative techniques for mutation detection should be considered (18).

Patients with advanced or metastasis carcinomas and bronchioloalveolar carcinomas bearing mutations in tyrosin-kinase domain can benefit from biological therapy. These patients can therefore be considered for receiving the biological therapy in II. line and be spared negative side effects of further chemotherapy. As a result of low DNA yields isolated from paraffin sections, the cytology smears seem preferable for specimen examination.

Table I: An overview of EGFR somatic mutations found in 23 gefitinib responders from Czech population of adenocarcinoma and BAC patients.

Exon 18	**G724G**
Exon 19	**del745-750insK** **del746-751insA** **del747-751insS**
Exon 20	**T790I**
Exon 21	**-**
Exon 22	**-**
Exon 23	**-**

Figure 1: Detail of EGFR exon 18 sequence containing a mutation site; normal tissue (A), mutated tissue from a gefitinib responding patient (B).

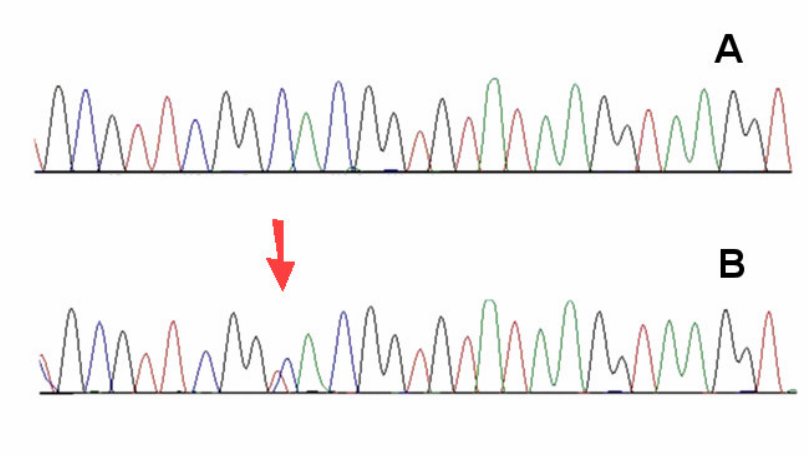
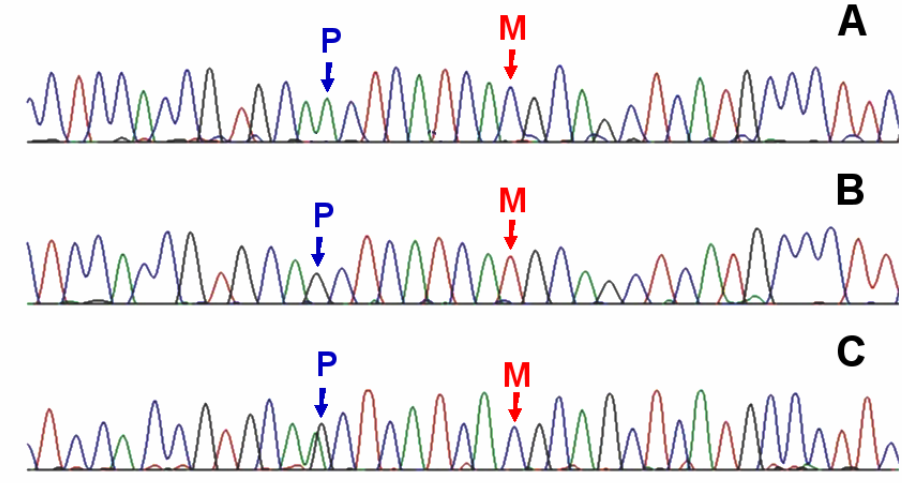


Figure 2: Detail of EGFR exon 20 sequence containing mutation site (marked with "M") and a polymorphic site (marked with "P"); normal tissue with A/A genotype (A), mutant tissue with G/G genotype and normal tissue with A/G genotype.



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Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with allelic losses at 9p and 18q in EUS-guided fine needle aspiration samples of patients with chronic pancreatitis and pancreatic cancer

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Aim: EUS-guided fine needle aspiration cytology (FNA) is standard diagnostic procedure for evaluation of suspicious pancreatic mass. Genetic tests in DNA extracted from pancreatic FNAs have long been studied as potential markers of malignancy. In this work, we examine some of the most characteristic genetic changes occurring in pancreatic neoplasia from patients undergoing FNA biopsy to reveal their usefulness in routine clinical diagnostic testing. In addition to conventional FNA cytology we attempt to establish an optimum combination of molecular markers overall resulting in best diagnostic sensitivity and specificity.

Methods: 101 consecutive patients (63 males, 38 females, 60 ± 12 years; 81 with subsequently diagnosed pancreatic cancer, 20 with chronic pancreatitis) with focal pancreatic mass underwent EUS-guided FNA. Samples were evaluated by rapid H&E staining followed by more detailed evaluation using Giemsa staining method. DNA was extracted from Giemsa stained smears using laser microdissection and examined for the presence of K-ras, p53 and p16 somatic mutations by CGCE and SSCP techniques. In addition allelic losses of tumor suppressor genes p16 (INK4, CDKN2A) and DPC4 (MADH4, SMAD4) were detected by monitoring the loss of heterozygosity (LOH) at 9p and 18q, respectively.

Results: Sensitivity and specificity of EUS-guided FNA was 100% for 74% of evaluated samples. The remaining 26% samples were assigned as inconclusive. Testing the same group for molecular markers revealed sensitivity and specificity of 70% and 100% for K-ras mutations($p<0,001$), 24% and 90% for p53 mutations (NS), 13% and 100% for p16 mutations (NS), 85% and 64% for allelic losses at 9p ($p<0,001$) and 78% and 57% for allelic losses at 18q ($p<0,05$). When different molecular markers were combined, the best results were obtained with K-ras + LOH at 9p (92% and 64%, $p<0,001$), K-ras + LOH at 18q (92% and 57%, $p<0,001$), and K-ras + LOH 9q + LOH 18q (96% and 43%, $p<0,001$). When the molecular markers were used as complements to evaluate FNA cytology inconclusive samples only, the overall sensitivity of cancer detection was 100% in all patiens enrolled in the study.

Conclusion: EUS-guided FNA cytology combined with screening of K-ras mutations and allelic losses of tumor suppressors p16 and DPC4 represents a very sensitive method in screening for pancreatic malignancy. Molecular markers may find its use particularly in cases where FNA cytology has been inconclusive.

Key words: pancreatic cancer, chronic pancreatitis, EUS-guided FNA, molecular markers, K-ras, p53, p16, DPC4, LOH, cytology

Introduction:

Enormous progress in diagnostic and therapeutic approaches in the last decade had very limited impact on generally poor survival rates of patients diagnosed with pancreatic cancer (PC)[1]. Mortality of the disease is almost at the level of its incidence as the majority of cases is diagnosed in advanced, not resectable stage[2]. Since the fundamental molecular-genetic mechanisms of PC have already been recognized, there is a great expectation that molecular tests could substantially assist in early diagnosis as well as open new therapeutic possibilities for this serious disease[3,4].

The development of pancreatic cancer follows a distinct path from normal ductal epithelia, pancreatic intraepithelial neoplasia (PanIN I–III) up to the carcinoma[5,6]. This path is accompanied by sequential accumulation of genetic defects (mostly point mutations, gene amplifications and allelic deletions). Activation of K-ras oncogene by somatic point substitution is seen as an initial event in pancreatic carcinogenesis is [7]. This alteration can be detected already in PanIN-1A lesions as well as in chronic pancreatitis (CP) and therefore represents an independent risk factor for pancreatic cancer. In advanced pancreatic cancer, K-ras mutations are found in close to 90% of cases, therefore considered as a potential molecular marker for early detection of developing cancer. Following the initial K-ras activation, a number of other genetic abnormalities take place. PanIN-1A and PanIN-1B phases are characterized by overexpression of Her-2/neu oncogene, which is found in 50% of pancreatic neoplasms. Increased Her-2/neu expression, however, is a result of higher transcription rate rather than gene amplification, rendering Her-2/neu an unusable therapeutic target[8]. Aside from the above oncogenes, there is a number of tumor suppressor genes affected by genetic alterations during the transformation process. Among them, p16 tumor-suppressor (also referred to as CDKN2 or INK4), located at chromosome 9p21 is inactivated already during transition from PanIN-1B to PanIN-2 phases[9]. Furthermore, loss of another important tumor-suppressor gene, SMAD4 (known also as *deleted in pancreatic carcinoma,* DPC4), located at chromosome 18q21 has also been observed[10]. Consequently, the p16 and DPC4 are inactivated in almost 95% (55% respectively) of cases of invasive pancreatic cancers, therefore, potentially useable as molecular markers. All of the genetic mutation events adversely affect control of the cell cycle, thus enabling defected cells to proliferate. Oncogene K-ras encodes for GTP-binding protein responsible for signaling in MAP-kinase pathway of intracellular signal transduction[11]. Tumor suppressor gene p53 is translated into a product that regulates transcription of other regulatory proteins, such as p21, inhibitory protein of cyclinD/CDK2 family[12]. The product of p16 tumor suppressor binds to the complex of cyclinD/CDK4 or CDK6, and thus regulates progression of cell cycle at the G1 control point[13]. Finally, the DPC4 tumor suppressor is a member of SMAD protein family which play crucial role in intracellular signaling of TGF-beta[14].

Current diagnostic approaches mostly rely on evaluation of morphological changes in pancreatic tissue in combination with histology/cytology examination of samples obtained by fine-needle aspiration (FNA)[15]. EUS-guided FNA typically delivers sensitivity of 80% and and specificity of 99%, while its positive and negative predictive values are at 99%, and 73% levels[16]. In order to increase diagnostic sensitivity of the FNA cytology, several papers have demonstrated detection of somatic aberrations as potential markers for early pancreatic cancer in DNA material from pancreatic juice, pancreatic ductal brushings, peroperative or percutaneous biopsies, plasma, duodenal aspirate, bile or stool. Among the various molecular markers in pancreatic cancer, K-ras is the most frequently studied. Its prevalence is estimated to reach 90–95%. The

reported rates of positivity, however, depend on experimental method of K-ras mutation detection as well as on the source material in which presence of K-ras mutations is to be detected. The capture rates range from 78–100% in pancreatic tissue[17], 61–89% in pancreatic juice[18,19], 72–83% in pancreatic ductal brushing[20,21], 35% in plasma[22], 33% in bile[23], and 25% in duodenal aspirate[24]. Detection of K-ras in stool gives better sensitivity than in bile, however specificity drops singificantly[25]. Acceptable specificity was reported only in pancreatic ductal brushings and pancreatic juice (77–100%).

Because of the high sensitivity of genetic testing in pancreatic juice, numerous mutations in other genes have been reported in this material. Sensitivity and specificity of genetic tests in pancreatic juice is 40–89% and 33–96% for K-ras[26,27], 11–43% and 70–100% for p16[18,28,29], 14–47 % and 88–100% for p53[25,30], 36–70% and 39–100% for DPC4[18,31]. The combination of several molecular markers in pancreatic juice is believed to improve sensitivity of genetic testing, giving best results for combination K-ras plus p53 which resulted in 100% sensitivity[30]. In contrast to pancreatic juice analysis there is only a limited number of publication on frequency of gene mutations in EUS-guided FNA samples. Takahashi´s study which included 62 consecutive patients with focal pancreatic mass is the largest. The authors screened for K-ras mutations and gave 74% sensitivity with 100% specificity[32].

From the original PC progression model it is clear that the pancreatic malignant conversion comes from a combination of multiple genetic events rather than originating from a single mutation[3,5]. Given the inherent heterogeneity of the carcinogenic pathways, simultaneous examination of multiple markers should lead to improved testing efficacy. The aim of the presented work was to evaluate a possibility of examining several somatic genetic events as potential molecular markers for early detection of pancreatic cancer in risk groups, such as in chronic pancreatitis patients. The main emphasis was on finding an ideal combination of markers bringing the optimum results when used in combination with commonly used cytology readings.

Materials and methods:

106 consecutive patients with focal pancreatic mass undergoing EUS-guided fine needle aspiration (FNA) were enrolled into the study. Patients were divided into pancreatic cancer group and the control group of patients with chronic pancreatitis based on histology of surgical specimen or long term follow-up. Five patients were excluded due to other diagnosis (adenoma, malignant fibrous histiocytoma, endocrine tumor, cholangiogenic carcinoma), or for malignant duplicity (patient with a breast carcinoma).

Of 101 patients in the final group a total of 63 (62%) were males and 38 (38%) females. The mean age in the group was 60±12 years, range 32–84 years (+2,01/-2,21 standard deviation). There were 81 (80%) patients with pancreatic cancer, and 20 (20%) patients with chronic pancreatitis. All patients signed informed consent with participation in the study as well as with genetic analysis of their tissue material.

EUS was performed by a single experienced endoscopist using GFUM-20 radial and GFUCT-140 linear array scanning echoendoscopes (Olympus Europe). Quality of FNA samples was evaluated by on-site cytologist after quick staining by hematoxylin-eosin. Definitive FNA diagnosis was stated by a single pathologist, blinded to the EUS, after staining additional slides by Giemsa. The same samples were subsequently submitted for genetic analysis. Furthermore, in a subset of 18 patients the genetic analysis of FNA sample was extended to genetic analysis of tissue acquired during subsequent peroperation biopsy. Laser microdissection of Giemsa-positive cells was performed on P.A.L.M. Microlaser instrument (Carl Zeiss,

Germany). Normally, between 100 and 200 cells were dissected from each slide. Genomic DNA was extracted from the dissected cells by a standard spin-column extraction protocol using GMC tissue DNA isolation kit (Genomac, Czech Republic).

Presence of somatic point mutations in codons 12 and 13 of K-ras and in exons 5–8 of p53 was detected by cycling-gradient capillary electrophoresis (CGCE), a high-sensitivity mutation detection technique based on heteroduplex analysis in temperature gradient[33]. The experimental details of the K-ras and p53 mutation assay were described previously[34,35]. Briefly, a PCR amplification of the target sequence containing the mutation hotspots was performed with one of the primers fluorescently labeled and the other primer extended by a 40bp artificial high-melting domain (GC-clamp). Following PCR, the ~140 bp fragment was heated and slowly cooled to allow formation of homo- and heteroduplex forms upon re-annealing of wildtype and mutant sequences. The resulting double-stranded fragments were subjected to capillary-electrophoretic separation at cycling temporal temperature gradient. A typical result is shown in Figure 1.

Mutations in p16 gene were analyzed by standard single-strand conformation polymorphism (SSCP) using amplification conditions previously described in literature[36,37] followed by capillary electrophoresis analysis of the resulting fragments in non-denaturing gel matrix (GMC-SSCP, Genomac, Czech Republic).

Allelic losses at chromosomal positions 9p and 18q were monitored by the loss of heterozygosity analysis (LOH) using a total of 3 microsatellite (STR) markers for 9p (D9S157, D9S171 and D9S1748) and 2 markers for 18q (D18S363, D18S474)[38,39]. Detected LOH at chromosomal site 9p is shown in Figure 2.

All capillary electrophoretic experiments including previously described temperature-gradient, SSCP and fragment analysis were performed on capillary-array DNA sequencer (MegaBACE™ 1000, GE Healthcare, Piscataway, NJ) equipped with Caddy™ 1000 robotic sample loader (Watrex Praha, Prague, Czech Republic) for unattended overnight operation.

Statistical analysis was based on two-way and multiway contingency tables, sensitivity and specificity of tests and on 95% confidence interval of relative frequencies with use of BMDP PC90 and MedCalc software.

Results:

The data in the study represent patients collected within a 2-year period from 2003 – 2005. Due to a dismal nature of the disease the total project time period exceeded by far the mean survival rate of pancreatic cancer patients enrolled in this study. The ultimate diagnosis of the malignant disease could, therefore, be unequivocal assigned based on clinical follow-up. During the final statistical analysis and evaluation, sensitivity and specificity of various diagnostic approaches performed during the patients dispensarisation could later be accurately determined.

Clinical examinations

Endoscopic ultrasonography (EUS)

EUS is considered the most sensitive method for visualising focal pancreatic lesions and staging of locoregional progression of the pancreatic disease[40]. All patients in our group were subjected to EUS for initial evaluation of pancreatic lesions. The EUS differentiation between malignant or benign nature of the

lesions resulted in 94% sensitivity and 77% specificity. The overall rate of false negatives was 5% and false positives 4%. In the remaining 11% endoscopist was not able to reliably state the diagnosis.

Fine-needle aspiration cytology (FNA-cytology)

EUS-guided FNA cytology has been adopted as a routine method for all patients admitted to our gastroenterology department with a suspicion of pancreatic cancer. The mail benefit of this safe method is in its sensitivity. At the same time, the acquired morphological information (TN staging) removes a need for additional diagnostic testing and/or surgery, surpassing CT or MR imaging [41]. Following the initial test for specimen quality by a "quick-test" using hematoxylin-eosin staining immediately following the puncture, samples were stained by Giemsa and thoroughly evaluated by an experienced cytologist. In the present study the overall sensitivity as well as specificity for FNA evaluated samples reached 100% with no malignant specimens assigned as benign or vice versa. In FNA testing, however, a 26% of smears were assigned as inconclusive. This mostly owing to the fact that the cellular atypia found in ductal epithelia did not allow clear differentiation between both diagnoses.

Histology of surgical resection tissue

Finally, in a subset of 18 patients, surgery was performed and collected pancreatic tissue was evaluated by pathologist. Histological evaluation of surgical resecate resulted ultimately in 100% specificity, but 95% sensitivity due to the fact that one resection sample was falsely evaluated as cancer-negative.

Molecular marker examination

Activating mutations in K-ras oncogene were found in 57 out of the total 101 samples. Comparison with the final diagnosis revealed that all K-ras positives were subsequently confirmed with malignancy, while none of the chronic pancreatitis samples exhibited K-ras mutation (Table 1). Hence, the resulting specificity was 100% and the sensitivity 70% with 95% CI (60%–80%). There were 24 (30%) cancerous specimens without K-ras mutation. Detecting mutations in tumor suppressor gene p53 uncovered only 19 positive cases (total of 101 cases) with a sensitivity of 24% with 95% CI (14%–32%) and specificity of 90% with 95% CI (85%–95%) (Table 2). Similarly, low mutation rates were obtained for p16 gene with 10 of 100 cases leading to only 13% sensitivity (95% CI 5%–9%), specificity was 100% (Table 3). 44 of the total of 66 samples exhibited allelic loss at 9p with the sensitivity of 85% with 95% CI (75%–94%) and specificity of 64% with 95% CI (53%–75%) (Table 4). Although 9p harbors p16 tumor-suppressor gene, no correlation was found between occurrence of p16 mutations and 9p allelic deletions. A combination of the two tests (p16 mutations and losses at 9p) yielded overall sensitivity of 84% with the specificity of 64%, 95% CI (75%–94%) and (53%–75%) respectively (Table 5). Sole LOH test at chromosomal position 18q, corresponding to a loss of tumor suppressor gene DPC4, was detected in 38 of 63 cases with sensitivity of 78% (95% CI 66%–89%) and specificity of 57% (95% CI 45%–69%) (Table 6).

When combining tests for independent molecular markers, the best results were obtained with a combination of K-ras and LOH 9p (Table 7). Sensitivity and specificity of this combination were 92% with 95% CI (86%–99%) and 64% with 95% CI (53%–75%), respectively. Another promising combination was K-ras and LOH 18q (Table 8) resulting in sensitivity of 92% with 95% CI (85%–99%) and specificity of 57% with

95% CI (45%–69%). By combining two markers with high specificity K-ras and low-sensitivity p53 a reasonable values were obtained: sensitivity of 74% with 95% CI (65%–83%) and specificity of 90% with 95% CI (85%–95%) (Table 9). This, however, does not significantly improve the sole K-ras test showing 70% sensitivity and 100% specificity as noted above. Similarly, a combination of the LOH tests performed on the two chromosomal loci (9p and 18q) resulted in the sensitivity of 92% with 95% CI (84%–99%) and specificity of 43% with 95% CI (31%–55%) (Table 10), which does not surpass combination of the relatively simpler K-ras mutation test with either of the individual LOH tests.

If three markers are taken in account, a combination of K-ras with both LOH (18q and 9p) show the highest sensitivity of 96% with 95% CI (91%–100%) and specificity of 43% with CI (31%–55%) (Table 11). The combination of p53 with LOH 18q and 9p gives comparable results: sensitivity of 92% with 95% CI (85%–99%) and specificity of 43% with 95% CI (31%–55%) (Table 12). Combination of K-ras with both methods for detecting genetic variations in p16 (SSCP and LOH 9p) results in sensitivity of 92% with 95% CI (85%–99%) and specificity of 64% with 95% CI (53%–75%) (Table 13).

Finally, fidelity of genetic testing in FNA-cytology smears versus resection tissue was evaluated in case of 18 patients where both sample types were obtained. There was no discrepancy in K-ras, p53 or p16 mutation rates as the results were identical in both sample types. In contrary, however, FNA-cytology specimens proved to be more suitable for detection of allelic losses at 9p by LOH test (P <0,001); higher sensitivity of FNA specimens for detection of LOH 18q was close to statistical significance (P <0,10).

Discussion:

EUS-guided FNA-cytology is widely regarded as the “golden standard” in morphological diagnosis of the pancreatic neoplasms. In agreement with this common perception, our own experience also confirms a high diagnostic value of the technique with sensitivity and specificity reaching 100% over the course of the presented study. These results mirror high efficacy of the protocol if FNA biopsy is first evaluated on-site by the cytologist, and then conclusively interpreted by a skilled pathologist with proper experience in pancreatic cytology. This encouraging result, however, is reduced by the fact that in addition to the unequivocally assigned samples a remaining total of 26% of FNA smears are marked as inconclusive. This mirrors the fact that distincion between reactive changes in chronic pancreatitis and well differentiated adenocarcinoma may be problematic and cause underdiagnosis of pancreatic cancer[42]. Hence, the resulting 74% success rate of FNA-cytology clearly opens a need for additional diagnostic tools.

Molecular diagnosis of early pancreatic cancer has been studied since several years. Although many molecular markers have been identified, it is evident that diagnostic and/or screening should be based on a set of tests rather than relying on one universal molecular indicator. In our study, we have obtained reproducible results indicating a notable capture rate of pancreatic cancers by using a combination of multiple highly specific markers. As shown in Table 1, test for K-ras mutation exhibited the highest possible specificity. Our finding is in agreement with reports of K-ras testing in pancreatic juice (sensitivity of 40–89% and specificity 33–96%). With regard to K-ras testing in FNA samples, our sensitivity was similar to a recent study (70% vs. 74%), moreover, at the same time we have confirmed 100% specificity of K-ras test in FNA reported in the same study[32]. The fidelity of K-ras test in our work was followed by LOH analysis for the detection of allelic losses at 9p and 18q chromosomal positions (Tables 2 and 3). Satisfactory sensitivity with

relatively low specificity of all above mentioned genetic tests make them suitable for screening purposes rather than for differential diagnosis of pancreatic masses.

As expected, the LOH analysis greatly profitted from laser microdissection of tumor cells from FNA-cytology specimens. In comparison to manual dissection from resected tissue, the sensitivity for detection of allelic loss was higher for laser-microdissected FNA samples. Low diagnostic value of p53 and p16 point mutations is in agreement with the overall limits of sensitivity and specificity intervals for these markers being previously tested in pancreatic juice[18,25,28-30]. Similarly in FNA samples, p53 or p16 mutations seem suitable for differential diagnosis or screening in FNA samples.

Based on the observations from this study, a diagnostic algorithm reflecting the most efficient approach to distinguish pancreatic cancer from chronic pancreatitis in FNA samples can be constructed (see Figure 3). As the EUS-guided FNA-cytology still has the highest sensitivity and specificity, it should always remain a preferred method of choice for examination of a focal pancreatic mass (Figure 3, step 1). Only a subset of FNA-inconclusive samples should be further examined by genetic analyses. The size of such sample set will undoubtedly depend on the pathologist's level of expertise. Of the various markers, K-ras is a prime candidate for first-level genetic analysis as the K-ras positivity showed to reliable differentiate patients with malignancy (Figure 3, step 2). Because of a lower sensitivity of K-ras test, samples negative for K-ras mutation should, consequently, be examined for allelic losses by LOH tests. Due to its higher sensitivity, LOH on the chromosome 9p, should be tested first (Figure 3, step 3), followed by a final testing of the LOH 18q performed on the remaining samples showing negativity for all previous tests (Figure 3, step 4). Such a set of four subsequent testing steps delivers satisfactory results. When using to process data acquired in our study, malignancy was correctly assigned to all patients with ultimately confirmed cancer status with no false negatives. One patient with chronic pancreatitis was incorrectly assigned with pancreatic cancer, a false positive, due to positivity of both 9p and 18q LOH tests.

In conclusion, the most sensitive genetic test for screening for malignancy in EUS-guided FNA samples from pancreatic mass seems a combination of K-ras mutation analysis with detection of p16 gene loss by LOH at 9p. Combination of K-ras with LOH analysis at both p16 and DPC4 genes further improves the sensitivity to 96%. The best compromise of sensitivity and specificity according to the Youden´s index is single K-ras (0,70) or combination of K-ras with LOH 9p (0,57). Based on our observations it seems that due to relatively high specificity of the used markers, malignancy is usually indicated already by a single positive test. Therefore, only negative samples are subsequently tested by further markers, increasing the cost effectivity of such diagnostic testing.

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Figure 1. An example of the K-ras mutation analysis by CGCE method: **A** – sample without mutation, **B** – sample with k-ras mutation in codon 12

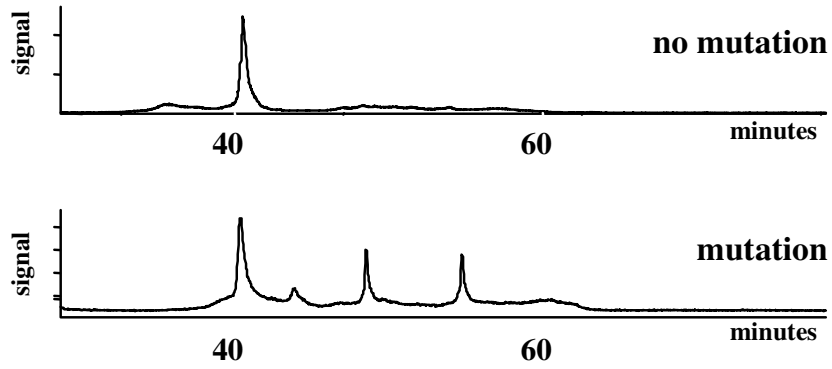

Figure 2. An example of LOH at chromosome 9p, analysis by CGCE method: **A** – tumor sample with allelic loss, **B** – blood sample with both alleles present

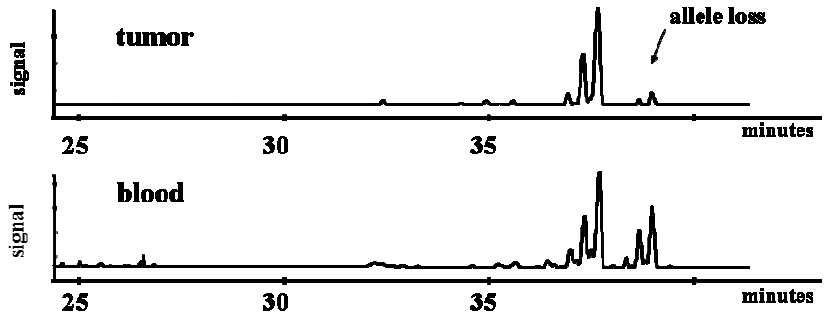

Figure 3. Four-step diagnostic algorithm evaluatin FNA cytology and genetic changes for differentiation benign and malignant lesions of the pancreas

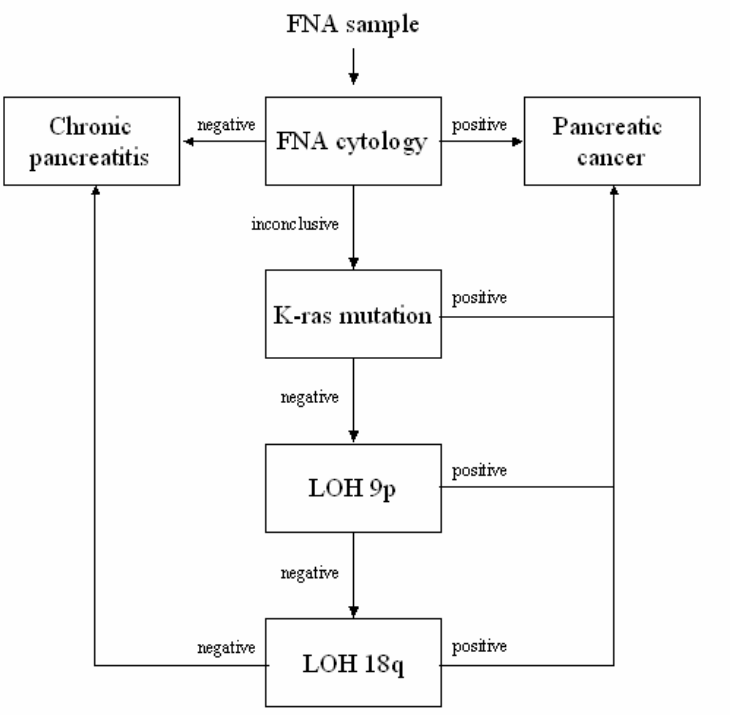

Table 1. Test for K-ras mutations.

frequency

	chronic panceratitis	pancreatic cancer	total
negative	20	24	44
positive	0	57	57
total	20	81	101

percentage

	chronic panceratitis	pancreatic cancer	total
negative	100%	29,6%	43,6%
positive	0%	70,4%	56,4%
total	100%	100%	100%

sensitivity: 70% 95% CI (60% – 80%)
specificity: 100%
Youden´s Index: 70%
p<0,001

Table 2. Test for p53 mutations.

frequency

	chronic panceratitis	pancreatic cancer	total
negative	18	62	80
positive	2	19	21
total	20	81	101

percentage

	chronic panceratitis	pancreatic cancer	total
negative	90,0%	76,5%	79,2%
positive	10,0%	23,5%	20,8%
total	100%	100%	100%

sensitivity: 24% 95% CI (14% – 34%)
specificity: 90% 95% CI (85% – 95%)
Youden´s Index: 14%
p=0,18 (NS)

Table 3. Test for p16 mutations.

frequency

	chronic panceratitis	pancreatic cancer	total
negative	20	70	90
positive	0	10	10
total	20	80	100

percentage

	chronic panceratitis	pancreatic cancer	total
negative	100%	87,5%	90,0%
positive	0%	12,5%	10,0%
total	100%	100%	100%

sensitivity: 13% 95% CI (7% –19%)
specificity: 100%
Youden´s Index: 13%
p=0,096 (NS)

Table 4. Test for LOH at chromosome 9p (site of p16 gene).

frequency

	chronic panceratitis	pancreatic cancer	total
negative	9	8	17
positive	5	44	49
total	14	52	66

percentage

	chronic panceratitis	pancreatic cancer	total
negative	64,3%	15,4%	25,8%
positive	35,7%	84,6%	74,2%
total	100%	100%	100%

sensitivity: 85% 95% CI (75% – 95%)
specificity: 64% 95% CI (53% – 75%)
Youden´s Index: 49%
p<0,001

Table 6. Test for LOH at chromosome 18q (site of DPC4 gene).

frequency

	chronic panceratitis	pancreatic cancer	total
negative	8	11	19
positive	6	38	44
total	14	49	63

percentage

	chronic panceratitis	pancreatic cancer	total
negative	57,1%	22,4%	30,2%
positive	42,9%	77,6%	69,8%
total	100%	100%	100%

sensitivity: 78% 95% CI (67% – 89%)
specificity: 57% 95% CI (45% – 69%)
Youden´s Index: 35%
p<0,05

Table 5. Combination of tests p16 + LOH 9p.

chronic pancreatitis:

	LOH 9p negative	LOH 9p positive	total
p16 negative	9	5	14
p16 positive	0	0	0
total	9	5	14

pancreatic cancer:

	LOH 9p negative	LOH 9p positive	total
p16 negative	8	36	44
p16 positive	0	7	7
total	8	43	51

sensitivity: 84% 95% CI (74% – 94%)
specificity: 64% 95% CI (53% – 75%)
Youden´s Index: 49%
p<0,001

Table 9. Combination of tests K-ras + p53.

chronic pancreatitis:

	p53 negative	p53 positive	total
K-ras negative	18	2	20
K-ras positive	0	0	0
total	18	2	20

pancreatic cancer:

	p53 negative	p53 positive	total
K-ras negative	21	3	24
K-ras positive	41	16	57
total	62	19	81

sensitivity: 74% 95% CI (65% – 83%)
specificity: 90% 95% CI (85% – 95%)
Youden´s Index: 64%
p<0,001

Table 8. Combination of tests K-ras + LOH 18q.

chronic pancreatitis:

	LOH 18q negative	LOH 18q positive	total
K-ras negative	8	6	14
K-ras positive	0	0	0
total	8	6	14

pancreatic cancer:

	LOH 18q negative	LOH 18q positive	total
K-ras negative	4	10	14
K-ras positive	7	28	35
total	11	38	49

sensitivity: 92% 95% CI (85% – 99%)
specificity: 57% 95% CI (45% – 69%)
Youden´s Index: 49%
p<0,001

Table 7. Combination of tests K-ras + LOH 9p.

chronic pancreatitis:

	LOH 9p negative	LOH 9p positive	total
K-ras negative	9	5	14
K-ras positive	0	0	0
total	9	5	14

pancreatic cancer:

	LOH 9p negative	LOH 9p positive	total
K-ras negative	4	11	15
K-ras positive	4	33	37
total	8	44	52

sensitivity: 92% 95% CI (85% – 99%)
specificity: 64% 95% CI (53% – 75%)
Youden´s Index: 57%
p<0,001

Table 10. Combination of tests LOH 9p + LOH 18q.

chronic pancreatitis:

	LOH 18q negative	LOH 18q positive	total
LOH 9p negative	6	3	9
LOH 9p positive	2	3	5
total	8	6	14

pancreatic cancer:

	LOH 18q negative	LOH 18q positive	total
LOH 9p negative	4	4	8
LOH 9p positive	7	32	39
total	11	36	47

sensitivity: 92% 95% CI (85% – 99%)
specificity: 43% 95% CI (31% – 55%)
Youden´s Index: 34%
p<0,01

Table 11. Combination of tests LOH 18q + LOH 9p + K-ras.

	LOH 18q	LOH 9p	K-ras		total
chronic pancreatitis (n = 14)	negative	negative	6	0	6
		positive	2	0	2
		total	8	0	8
	positive	negative	3	0	3
		positive	3	0	3
		total	6	0	6
pancreatic cancer (n = 49)	negative	negative	2	2	4
		positive	2	5	7
		total	4	7	11
	positive	negative	2	2	4
		positive	8	26	34
		total	10	28	38
total					63

sensitivity: 96% 95% CI (92% – 100%) false positive: 8
specificity: 43% 95% CI (31% – 55%) false negative: 2
Youden´s Index: 39%
p<0,001

Table 12. Combination of tests LOH 18q + LOH 9p + p53.

	DPC4 (LOH)	p16 (LOH)	p53		celkem
chronic pancreatitis (n = 14)	negative	negative	6	0	6
		positive	2	0	2
		total	8	0	8
	positive	negative	3	0	3
		positive	2	1	3
		total	5	1	6
pancreatic cancer (n = 49)	negative	negative	4	0	4
		positive	4	3	7
		total	8	3	11
	positive	negative	4	0	4
		positive	26	8	34
		total	30	8	38
total					63

sensitivity: 92% 95% CI (85% – 99%) false positive: 8
specificity: 43% 95% CI (31% – 55%) false negative: 4
Youden´s Index: 35%
p<0,001

Table 13. Combination of tests p16 + LOH 9p + K-ras.

	p16 (SSCP)	p16 (LOH)	K-ras		celkem
chronic pancreatitis (n = 14)	negative	negative	9	0	9
		positive	5	0	5
		total	14	0	14
	positive	negative	0	0	0
		positive	0	0	0
		total	0	0	0
pancreatic cancer (n = 51)	negative	negative	4	4	8
		positive	8	28	36
		total	12	32	44
	positive	negative	0	0	0
		positive	2	5	7
		total	2	5	7
total					65

sensitivity: 92% 95% CI (85% – 99%)
specificity: 64% 95% CI (53% – 75%)
Youden´s Index: 57%
p<0,001

false positive: 5
false negative: 4

5 Závěr

V prezentovaných pracích jsme se zabývali vývojem a praktickou aplikací metod pro rychlou, citlivou, vysokokapacitní detekci molekulárních markerů u různých typů nádorových onemocnění. Vyvinuli jsme v této souvislosti dvě nové metodiky. První je založená na cyklujícím teplotním gradientu, který poskytuje efektivnější separaci DNA molekul a možnost opakovaného dávkování v jedné analýze a tedy výrazné zvýšení celkového počtu analyzovaných vzorků [1]. Druhá metodika založená na dynamickém značení DNA molekul interkalačním činidlem během elektroforetické analýzy odstraňuje potřebu fluorescenčně značených primerů a dělá tak analýzy finančně i prakticky dostupnějšími [3]. Obě tyto metodiky jsme následně použili při analýzách klinických vzorků pacientů s nádorovým onemocněním. Výsledky uvedených publikací zaměřených na klinické aplikace ukazují, že vyšetření molekulárních markerů zahrnující mutace, alelické ztráty a jednonukleotidové polymorfismy má široké uplatnění.

Charakterizace vzorků kolorekta na molekulární úrovni, jehož optimalizaci jsme provedli, může mít význam při popisu stádia transformace adenomu v karcinom nebo při odhadu prognózy onemocnění na základě detekovaných typů mutací [2]. U pacientů s podezřením na karcinom pankreatu může molekulárně-biologické vyšetření v kombinaci s cytologickým zpřesnit diagnózu, což je zejména v časných stádiích onemocnění pro včasný chirurgický zákrok zásadní [8]. Molekulární markery ale mohou být také důležité při odhadu odpovědi na určitý typ léčby, jak vyplývá z našich výsledků analýz, které jsme provedli na vzorcích nemalobuněčného karcinomu plic v souvislosti s biologickou léčbou inhibitorem tyrozin-kináz [7]. V některých případech tento typ analýz také napomáhá ke zjišťování původu nádoru, jak jsme ukázali u mnohočetného karcinomu močového měchýře, kde naše výsledky podpořily teorii o monoklonálním původu tohoto karcinomu [4]. V neposlední řadě má vyšetření molekulárních markerů, konkrétně jednonukleotidových polymorfismů, uplatnění při určení rizikových jedinců se zvýšenou pravděpodobností výskytu nádorového onemocnění. V našem případě se jednalo o karcinom prostaty, kde se nám podařilo nalézt minimálně jeden SNP, u něhož jedna z alel je statisticky významně zvýšená u pacientů s tímto typem nádoru a je tedy potenciálním kandidátem pro preventivní screening [5,6].

Nádorová onemocnění představují jeden z nejkomplexnějších problémů řešených v současném oboru přírodních věd. Jedním z důvodů je vysoká rozmanitost molekulárních

mechanismů, které vedou ke vzniku a progresi nádorů, dalším důvodem je i obecná nádorová heterogenita, kde morfologicky stejné tumory vykazují rozdílné histopatologické chování jakým je například agresivita, resistence vůči cytostatikům atd. Výzkum základních mechanismů na úrovni DNA variací (mutací a polymorfismů) se v současnosti ukazuje jako nejvhodnější pro časnou detekci specifických pochodů a profilovaní aktuálního stavu dění v postižené tkáni na molekulární úrovni. Věřím, že i výsledky projektů popsaných v předkládané práci přispějí alespoň drobným dílem k poznání a získání nového pohledu na toto závažné onemocnění.

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7 Prohlášení

Prohlašuji, že jsem tuto práci ani její podstatnou část nepředložila k získání jiného nebo stejného akademického titulu.

V Praze, dne 19.3.2007 Lucie Benešová

Prohlašuji, že se Lucie Benešová podílela na níže uvedených publikacích v tomto rozsahu:

Cycling gradient capillary electrophoresis: a low-cost tool for high-throughput analysis …
- optimalizace separačních podmínek pro analýzu vybraných markerů

Application of cycling gradient capillary electrophoresis to detection of APC, K-ras …
- návrh primerů pro APC a DCC markery, příprava vzorků pro analýzu (izolace DNA, optimalizace PCR podmínek, PCR), optimalizace separačních podmínek pro analyzované markery, sekvenace vybraných PCR produktů, vyhodnocení získaných dat

Multicapillary electrophoresis of unlabeled DNA fragments with high-sensitive laser-induced …
- spolupráce na provedených analýzách a vyhodnocení výsledků

Parallel optimization and genotyping of multiple single-nucleotide polymorphism markers …
- spolupráce na návrhu primerů pro jednotlivé markery, optimalizaci PCR podmínek, analýze markerů a vyhodnocení získaných dat

Analysis of genetic events in 17p13 and 9p21 regions supports predominant monoclonal …
- optimalizace a následné provedení LOH analýz oblastí 17p13 a 9p21 a spolupráce na vyhodnocení získaných dat

Frekvence výskytu vrozených DNA polymorfismů genů syntetické dráhy testosteronu…
- spolupráce na návrhu primerů pro jednotlivé markery, optimalizaci PCR podmínek, analýze markerů a vyhodnocení získaných dat

Genetic variations in epidermal growth factor receptor gene (EGFR) and their role in prediction …
- izolace DNA, optimalizace PCR podmínek, optimalizace sekvenačních podmínek, vyhodnocení získaných dat

Evaluation of clinical relevance of examining K-ras, p16 and p53 mutations along with …
- optimalizace SSCP podmínek pro analýzu mutací v genu p16, návrh primerů pro PCR p53 marker (exon 7), LOH analýzy oblastí 17p13 a 9p21, vyhodnocení získaných dat

V Praze, dne 19.3.2007 RNDr. Marek Minárik, Ph.D.